

**THE ROLE OF METABOLISM IN DETERMINING
SUSCEPTIBILITY
TO PARATHION TOXICITY**

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candidates for the degree of**

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To the University of Newcastle-upon-Tyne

by

ELAINE MUTCH

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Department of Environmental and Occupational Medicine	The Medical School
NEWCASTLE UNIVERSITY LIBRARY	Framlington Place
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To Greg and Sally

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Publications

Mutch E, Blain PG and Williams FM (1996). Cytochrome P450 isozymes involved in parathion metabolism in the rat. Hum Exp Toxicol 15: 687.

Mutch E, Blain PG and Williams FM (1997). Cytochrome P450 isozymes involved in parathion metabolism by human liver. Hum Exp Toxicol 16: 409.

Mutch E, Blain PG and Williams FM (1998). The role of metabolism in determining human susceptibility to parathion toxicity. (Submitted to Xenobiotica).

CONTENTS

Abstract

List of figures

List of tables

Abbreviations

SECTION I

GENERAL INTRODUCTION

Page

Chapter 1. Organophosphate pesticides

1.1 Introduction	1
1.2 Organophosphate pesticides	1
1.3 Chemical structure and classification	2
1.4 Parathion	4

Chapter 2. The mode of action of organophosphates

2.1 Introduction	7
2.2 Acute toxic effects of organophosphates	7
2.2.1 Kinetics of acetylcholinesterase inhibition	8
2.2.2 Clinical features of acute toxicity	11
2.2.3 Biological monitoring for acute exposure to organophosphates	14
2.3 Delayed neurotoxic effects of organophosphates	15
2.4 Long term effects of organophosphates	16

Chapter 3. Xenobiotic metabolism

3.1 Introduction	20
3.2 The cytochrome P450 system	22

3.3	The flavin monooxygenase system	25
3.4	The esterases	27
3.4.1	The A-esterases	28
3.4.2	Cholinesterase	32
3.4.3	Acetylcholinesterase	36
3.4.4	Carboxylesterase	39
3.4.5	Neuropathy target esterase	47
3.5	Phase 2 enzymes	47
3.5.1	Glutathione-S-transferases	47
3.5.2	UDP glucuronosyltransferases and sulphotransferases..	48
3.6.	The proposed metabolism of parathion	50

Chapter 4. Pharmacogenetic polymorphisms

4.1	Introduction	53
4.2	Xenobiotic-mediated polymorphisms	56
4.2.1	The cytochrome P450 3A subfamily	61

Chapter 5. Factors influencing organophosphate toxicity

5.1	Introduction	66
5.2	Physicochemical properties	67
5.3	Physiological factors	68
5.3.1	Interethnic differences	68
5.3.2	Other modifying factors	72
5.4	Environmental factors	75
5.5	The route of absorption	84
5.6	Extrahepatic metabolism	88

Chapter 6. Experimental aims

6.1 Experimental aims	90
-----------------------	----	----	----	----	----	----

SECTION II
METHODS

Chapter 7. General methods

7.1 Chemicals	93
7.2 Animals	93
7.3 Buffer solutions	94
7.4 Preparation of rat and human liver subcellular fractions	95
7.5 Determination of the protein concentration of subcellular fractions	95
7.6 Spectral determination of the P450 content of rat liver microsomes	97
7.7 Statistical methods	98

Chapter 8. Biochemical methods

8.1 Measurement of the activity of P450s 1A and 2B	100
8.2 Measurement of the activity of P450 2E1 102
8.3 Measurement of the activity of P450 3A 104
8.4 Method for the study of parathion metabolism	.. 109
8.4.1 Results and discussion 115
8.5 Measurement of flavin monooxygenase activity	118
8.6 Measurement of paraoxonase activity 121
8.7 Measurement of phenylvalerate hydrolase activity	124
8.8 Measurement of glutathione-S-transferase activity	126

SECTION III

EXPERIMENTAL STUDIES

Chapter 9. Rat induction studies *in vivo*

9.1	Introduction	129
9.2	Methods	130
9.2.1	The induction regimen			130
9.2.2	Biochemical methods			131
9.3	Results	133
9.3.1	Control enzyme activities			133
9.3.2	The effect of induction on physiological parameters	137
9.3.3	The effect of induction on the cytochromes P450 and esterases		139
9.3.4	The effect of induction on parathion metabolism	152
9.4	Discussion	156

Chapter 10. Rat inhibition studies *in vitro*

10.1	Introduction	164
10.2	Methods	166
10.2.1	Inhibition of nifedipine oxidation	..					166
10.2.2	Inhibition of parathion metabolism	..					167
10.3	Results	168
10.4	Discussion	178

Chapter 11. The kinetics of parathion metabolism by rat liver microsomes

11.1	Introduction	181
------	--------------	----	----	----	----	----	-----

11.2	Methods	184
11.2.1	The effect of ketoconazole on parathion kinetics	185
11.3	Results	188
11.4	Discussion	198

Chapter 12. Parathion metabolism by human liver microsomes

12.1	Introduction	202
12.2	Methods	202
12.2.1	The subjects	202
12.2.2	Biochemical characterisation of human liver microsomes	204
12.2.3	Parathion metabolism by human liver microsomes	206
12.2.3.1	The kinetics of parathion metabolism	206
12.2.3.2	Inhibition of parathion metabolism	207
12.2.4	Inhibition of paraoxonase activity	208
12.2.5	Correlations between parathion metabolism and P450 marker activities	209
12.2.6	Studies with P450-expressed microsomes	209
12.3	Results	210
12.3.1	Biochemical characterisation of human liver microsomes	210
12.3.2	Interindividual variations in parathion metabolism	213
12.3.3	The kinetics of parathion metabolism	214
12.3.4	Inhibition of parathion metabolism	222

12.3.5	Inhibition of paraoxonase activity	..	228
12.3.6	Correlations between parathion metabolism and P450 marker activities		230
12.3.7	Studies with P450-expressed microsomes		237
12.4	Discussion	238

SECTION IV
GENERAL DISCUSSION

Chapter 13. General discussion

13.1	General discussion	245
13.2	Future studies	254

SECTION V
APPENDICES and REFERENCES

Chapter 14. Appendices

14.1	Appendix I	Data for rat inhibition study	..	256
14.2	Appendix II	Human liver microsomal recovery		260
14.3	Appendix III	Individual data for human liver enzymes	261
14.4	Appendix IV	Data for human inhibition study		269
14.5	Appendix V	Chromatograms for parathion and nifedipine metabolism	..	273

Chapter 15. References

15.1	References	275
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The role of metabolism in determining susceptibility to parathion toxicity Elaine Mutch (1998)

Abstract

Phosphorothioate insecticides such as parathion (O, O, diethyl O-*p*-nitrophenyl phosphorothioate) must undergo metabolic activation to form oxygen analogs in order to exert their toxicity. The specific isoforms of cytochrome P450 involved in this oxidative desulphuration reaction were examined in rat liver microsomes and a panel of sixteen human liver microsomes.

In the rat, parathion (20 μ M and 200 μ M) was activated to paraoxon with an apparent K_m of $10.4 \pm 0.25 \mu\text{M}$ ($n=3$), the metabolic rates were 241 ± 17 and 256 ± 18 pmol/min/mg protein, respectively. *p*-Nitrophenol was also formed, at 235 ± 15 and 220 ± 23 pmol/min/mg protein. Human liver microsomes activated parathion (20 μ M and 200 μ M) with an apparent K_m of 9 μ M-16 μ M ($n=3$), the metabolic rates were 23.3-199.3 and 18.7-310.3 pmol/min/mg protein ($n=16$). *p*-Nitrophenol was also formed, at 321.1-769.2 and 406.2-778.3 pmol/min/mg protein.

The activation of parathion (200 μ M) by human liver microsomes was positively correlated with nifedipine oxidation, indicating the involvement of CYP3A. Correlations were not significant with ethoxyresorufin-O-dealkylation, pentoxyresorufin-O-dealkylation, *p*-nitrophenol hydroxylation, paraoxon hydrolysis or phenylvalerate hydrolysis. Paraoxon formation from parathion by human liver microsomes was markedly inhibited by the CYP3A inhibitors ketoconazole, quercetin and naringenin (apparent $K_i=21 \mu\text{M}$). Metyrapone and α -naphthflavone had some inhibitory effect. The inhibitors were generally less effective towards parathion metabolism by rat liver microsomes. Experiments with EDTA indicated that A-esterase was not functionally important at low levels of paraoxon. Human P450s 3A4 and 3A5 expressed microsomes were the most efficient at biotransforming parathion to paraoxon, although P450s 1A1, 2B6 and 2C8 also catalysed the reaction.

The present study has shown marked interindividual variation in the metabolism of parathion, which may influence toxicity following exposure to this or other phosphorothioates. Co-administration of inhibitors or inducers of the enzymes involved may affect the fate of parathion and thus enhance or reduce its toxicity.

List of Figures

Figure		Page
1.1	The general chemical structure of phosphorothioates (and their oxons) ..	4
1.2	The structure of parathion	5
2.1	Reaction of paraoxon with acetylcholinesterase resulting in phosphorylated enzyme	10
2.2	Inhibition of an esterase system by organophosphate compounds ..	13
3.1	The central role of metabolism in the excretion of metabolites	21
3.2	Hydrolysis of acetylcholine by acetylcholinesterase resulting in reactivated enzyme	38
3.3	The general mechanism for hydrolysis by carboxylesterases	40
3.4	The phosphorylation of carboxylesterase by chlorpyrifos oxon	43
3.5	The hydrolysis of malathion by carboxylesterases	45
3.6	The proposed metabolism of parathion	51
4.1	Enzymes that demonstrate polymorphic expression in the human population ..	55
4.2	The relationship between debrisoquine metabolic ratio and genotype	58
5.1	An outline of the enzymes which may be involved in parathion metabolism in the rat and man	85
8.1	An Eadie Hofstee plot showing nifedipine oxidation by control rat liver microsomes	105

Figure		Page
8.2	Production of M1 from nifedipine (100μM) by control rat liver microsomes: the effect of varying time	106
8.3	Production of M1 from nifedipine (100μM) by control rat liver microsomes: the effect of varying protein concentration ..	107
8.4	A typical M1 extracted standard curve ..	110
8.5	Production of <i>p</i> -nitrophenol and paraoxon from parathion (200μM) by human liver microsomes: the effect of varying time	112
8.6	Production of <i>p</i> -nitrophenol and paraoxon from parathion (200μM) by human liver microsomes: the effect of varying protein concentration	113
8.7	A typical paraoxon standard curve ..	116
8.8	A typical <i>p</i> -nitrophenol standard curve	117
8.9	Paraoxonase (1mM) activity of human liver microsomes at 30°C: the effect of varying pH	122
9.1	Spectral P450 content following pretreatment with inducers	140
9.2	Ethoxyresorufin-O-dealkylase activity of rat liver microsomes: the effect of inducers	141
9.3	Pentoxyresorufin-O-dealkylase activity of rat liver microsomes: the effect of inducers	142
9.4	<i>p</i> -Nitrophenol hydroxylase activity of rat liver microsomes: the effect of inducers	143
9.5	Nifedipine oxidase activity of rat liver microsomes: the effect of inducers ..	144

Figure		Page
9.6	Rat liver paraoxonase activity (pH 9.0) expressed per mg protein: the effect of inducers	146
9.7	Rat liver paraoxonase activity (pH 9.0) expressed per g liver: the effect of inducers	147
9.8	Rat liver paraoxonase activity (pH 7.4) expressed per mg protein: the effect of inducers	148
9.9	Rat liver paraoxonase activity (pH 7.4) expressed per g liver: the effect of inducers	149
9.10	Rat liver phenylvalerate hydrolase activity expressed per mg protein: the effect of inducers	150
9.11	Rat liver phenylvalerate hydrolase activity expressed per g liver: the effect of inducers	151
9.12	The effect of inducers on paraoxon formation from parathion by rat liver microsomes	153
9.13	The effect of inducers on <i>p</i> -nitrophenol formation from parathion by rat liver microsomes	154
9.14	The effect of inducers on paraoxon and <i>p</i> -nitrophenol formation from parathion by rat liver microsomes. Data is expressed as the paraoxon/ <i>p</i> -nitrophenol ratio ..	155
10.1	Nifedipine oxidase (100μM) activity of control rat liver microsomes: the effect of inhibitors	169
10.2	Nifedipine oxidase (5μM and 100μM) activity of control rat liver microsomes: the effect of inhibitors	170

Figure		Page
10.3	Production of paraoxon from parathion by rat liver microsomes: the effect of inhibitors (1mM)	172
10.4	Production of <i>p</i> -nitrophenol from parathion by rat liver microsomes: the effect of inhibitors (1mM)	173
10.5	Production of paraoxon from parathion (200μM) by rat liver microsomes: the effect of inhibitors (100μM)	174
10.6	Production of <i>p</i> -nitrophenol from parathion (200μM) by rat liver microsomes: the effect of inhibitors (100μM)	175
10.7	Production of paraoxon from parathion (20μM) by rat liver microsomes: the effect of inhibitors (100μM)	176
10.8	Production of <i>p</i> -nitrophenol from parathion (20μM) by rat liver microsomes: the effect of inhibitors (100μM)	177
11.1	Production of <i>p</i> -nitrophenol and paraoxon from parathion (10μM) by rat liver microsomes: the effect of varying time	186
11.2	Production of <i>p</i> -nitrophenol and paraoxon from parathion (10μM) by rat liver microsomes: the effect of varying protein concentration	187
11.3	A Michaelis-Menten plot of paraoxon and <i>p</i> -nitrophenol formation by control rat liver microsomes	189
11.4	A Michaelis-Menten plot of paraoxon and <i>p</i> -nitrophenol formation by control rat liver microsomes pre-incubated with ketoconazole (5μM)	190
11.5	Computer fitted Michaelis-Menten plots of paraoxon and <i>p</i> -nitrophenol formation by non-inhibited rat liver microsomes	192

Figure		Page
11.6	An Eadie Hofstee plot of paraoxon formation by control rat liver microsomes	193
11.7	An Eadie Hofstee plot of paraoxon formation by control rat liver microsomes pre-incubated with ketoconazole (5 μ M)	194
11.8	An Eadie Hofstee plot of <i>p</i> -nitrophenol formation by control rat liver microsomes	195
11.9	An Eadie Hofstee plot of <i>p</i> -nitrophenol formation by control rat liver microsomes pre-incubated with ketoconazole (5 μ M)	196
12.1	A frequency distribution histogram for the formation of MI from nifedipine by 16 human liver microsomes	212
12.2	Paraoxon and <i>p</i> -nitrophenol formation from parathion (20 μ M) by 16 human liver microsomes: Individual paraoxon/ <i>p</i> -nitrophenol ratios	215
12.3	Paraoxon and <i>p</i> -nitrophenol formation from parathion (200 μ M) by 16 human liver microsomes: Individual paraoxon/ <i>p</i> -nitrophenol ratios	216
12.4	A Michaelis-Menten plot of paraoxon and <i>p</i> -nitrophenol formation by human liver microsomes M9	218
12.5	A Lineweaver-Burke plot of paraoxon formation by human liver microsomes M9	219
12.6	A Lineweaver-Burke plot of <i>p</i> -nitrophenol formation by human liver microsomes M9	220
12.7	An Eadie Hofstee plot of paraoxon and <i>p</i> -nitrophenol formation by human liver microsomes M9	221
12.8	Production of paraoxon from parathion (20 μ M) by human liver microsomes M15 and M16: the effect of inhibitors (100 μ M)	223

Figure		Page
12.9	Production of paraoxon from parathion (20μM) by human liver microsomes M9 and M10: the effect of inhibitors (100μM)	224
12.10	Production of paraoxon from parathion (200μM) by human liver microsomes M15 and M16: the effect of inhibitors (100μM)	225
12.11	Production of paraoxon from parathion (20μM) by human liver microsomes M9 and M10: the effect of inhibitors (100μM)	226
12.12	Dixon plot illustrating the inhibitory effect of naringenin on parathion activation to paraoxon by human liver microsomes (M11)	229
12.13	Correlation between parathion (200μM) and nifedipine (100μM) metabolism by 16 human liver microsomes	231
12.14	Correlation between parathion (20μM) and nifedipine (100μM) metabolism by 16 human liver microsomes	232
12.15	Correlation between parathion (200μM) and ethoxyresorufin (2μM) metabolism by 7 human liver microsomes	233
12.16	Correlation between parathion (200μM) and pentoxyresorufin (2μM) metabolism by 7 human liver microsomes	234
12.17	Correlation between nifedipine (100μM) and pentoxyresorufin (2μM) metabolism by 7 human liver microsomes	235

List of Tables

Table		Page
1.1	Several phosphorothioate pesticides used occupationally	3
2.1	A summary of acute toxicity features from organophosphate poisoning	12
2.2	Organophosphorus compounds shown to produce OPIDN in hens	17
3.1	A list of reactions classed as phase 1 or phase 2 metabolism	20
3.2	Sequential homology between rat and human P450 forms	25
3.3	Some environmental contaminant esters that are detoxified by the carboxylesterases	41
3.4	Some drugs that are detoxified by human carboxylesterases	41
4.1	Use or occurrence of chemicals metabolised by human hepatic cytochrome P450 3A4	64
5.1	Examples of substrates and inhibitors of the major human liver microsomal P450 isoforms involved in xenobiotic transformation	77
5.2	Examples and characteristics of five classes of inducers of rat liver microsomal cytochromes P450	81
9.1	Ethoxy- and pentoxyresorufin-O-dealkylase activities of control rat liver microsomal fractions	133
9.2	<i>p</i> -Nitrophenol hydroxylase activities of control rat liver microsomal fractions ..	134
9.3	Nifedipine oxidase activities of control rat liver microsomal fractions	134

Table		Page
9.4	Paraoxonase activity of control rat liver microsomal fractions measured at pH 7.4 and pH 9.0	135
9.5	Phenylvalerate hydrolase activities of control rat liver microsomal fractions ..	136
9.6	Parathion metabolism by control rat liver microsomal fractions	136
9.7	Body weights of rats at the beginning and end of the induction study	137
9.8	Liver weight, microsomal protein recovery and total P450 content of the microsomes at the end of the induction study	138
9.9	The effects of corn oil pre-treatment on various cytochromes P450 of rat liver ..	145
10.1	A list of the inhibitors/substrates used in the study and cytochromes P450 involved ..	165
11.1	The Vmax and apparent Km values for paraoxon and <i>p</i> -nitrophenol formation by control rat liver microsomes	197
11.2	The kinetic parameters for parathion metabolism by rat liver microsomes pre-treated with ketoconazole (5μM) <i>in vitro</i> .	197
12.1	Demographic details for human liver samples	203
12.2	Enzyme activities for the panel of human liver microsomal preparations	210
12.3	Parathion (20μM and 200μM) metabolism to paraoxon and <i>p</i> -nitrophenol by 16 human liver microsomal preparations	213
12.4	Kinetic parameters for paraoxon and <i>p</i> -nitrophenol formation from parathion by 3 human liver microsomal preparations	217

Table		Page
12.5	Inhibition of paraoxon formation from parathion by sulphaphenazole for 3 human liver microsomal preparations	227
12.6	Inhibition of <i>p</i> -nitrophenol formation from parathion by sulphaphenazole for 3 human liver microsomal preparations	228
12.7	The effect of EDTA on the metabolism of parathion and paraoxon by human liver microsomes	230
12.8	Correlations between enzyme activities and paraoxon and <i>p</i> -nitrophenol formation from parathion	236
12.9	Metabolic activity of microsomes prepared from human P450-expressed cell lines ..	237

Abbreviations

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CBC	Comparative biology centre
CYP	Cytochrome P450
DMSO	Dimethylsulphoxide
EC	Electron capture
EOR	Ethoxyresorufin-O-dealkylase
FID	Flame ionisation detection
FMO	Flavin monooxygenase
GC	Gas chromatography
GST	Glutathione-S-transferase
HPLC	High performance liquid chromatography
i.p.	Intra-peritoneal
i.v.	Intravenous
LD ₅₀	Mean lethal dose
NADPH	Nicotinamide adenine dinucleotide, reduced
OP	Organophosphate
PCN	Pregnenolone-16 α -carbonitrile
POR	Pentoxyresorufin-O-dealkylase
RNA	Ribose nucleic acid
sem	Standard error of the mean
s.c.	Subcutaneous

SECTION I:
GENERAL INTRODUCTION

Chapter 1

Chapter 1 Organophosphate pesticides

1.1 Introduction

Many thousands of different pesticides are used every year for home and agricultural purposes and consequently the potential for exposure to man is great. The term “pesticide” is a generic name for a variety of chemical substances used to kill or control undesirable plant or animal pests. These chemicals are designed to be toxic to certain organisms and are deliberately added to the environment. Even with advances in pest control, it is estimated that up to 50% of harvested crops can be damaged by post-harvest infestation by insects, fungi, rodents etc. Pesticides have thus become indispensable in feeding, clothing and protecting the worlds’ population.

1.2 Organophosphate Pesticides

Following the second world war, organochlorine pesticides made a major contribution to improvements in agricultural output and in the control of disease vectors. While the persistence of these compounds after application was of considerable benefit to the user, it also introduced problems. As these problems became more widely appreciated, insect pest control began to rely more on the anti-cholinesterase organophosphate and carbamate ester pesticides.

The insecticidal action of organophosphate compounds was observed in Germany during World War Two in the study of materials closely related to the nerve gases sarin, soman and tabun. Initially, the discovery was made in search of substitutes for nicotine, which was in very short supply

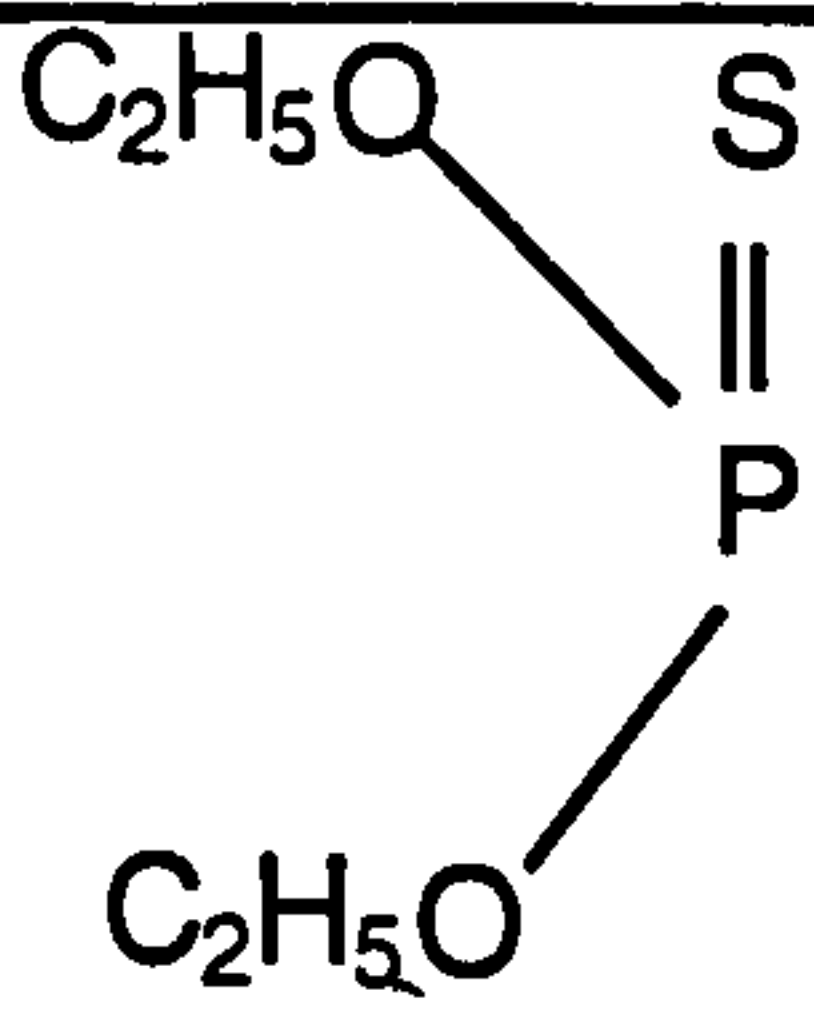
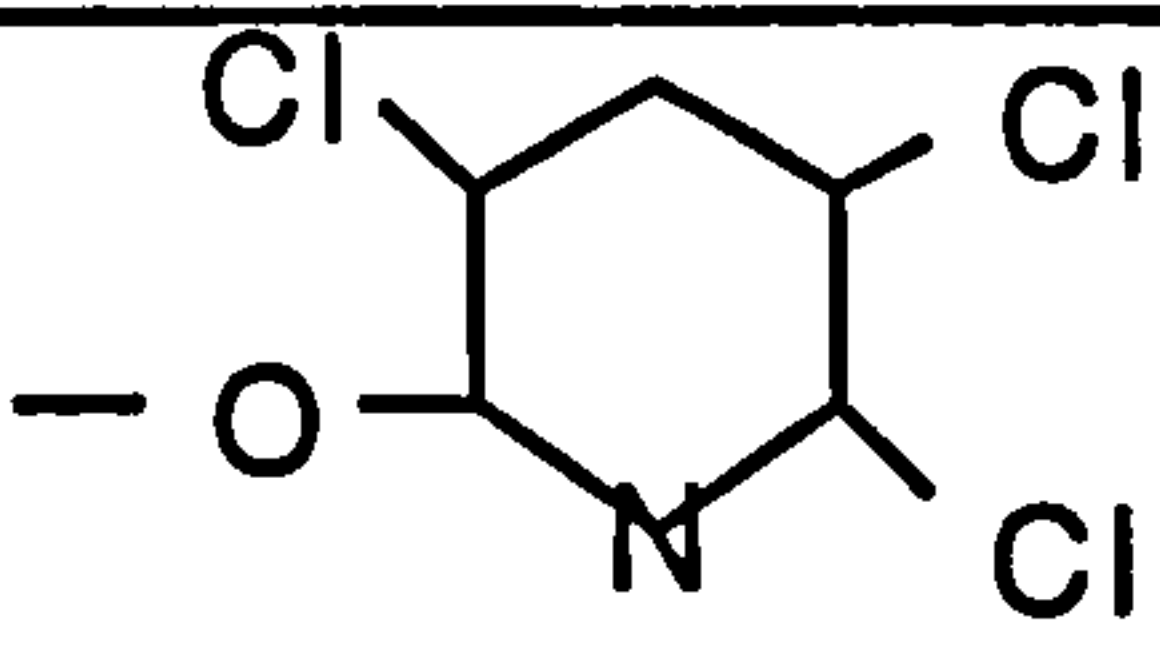
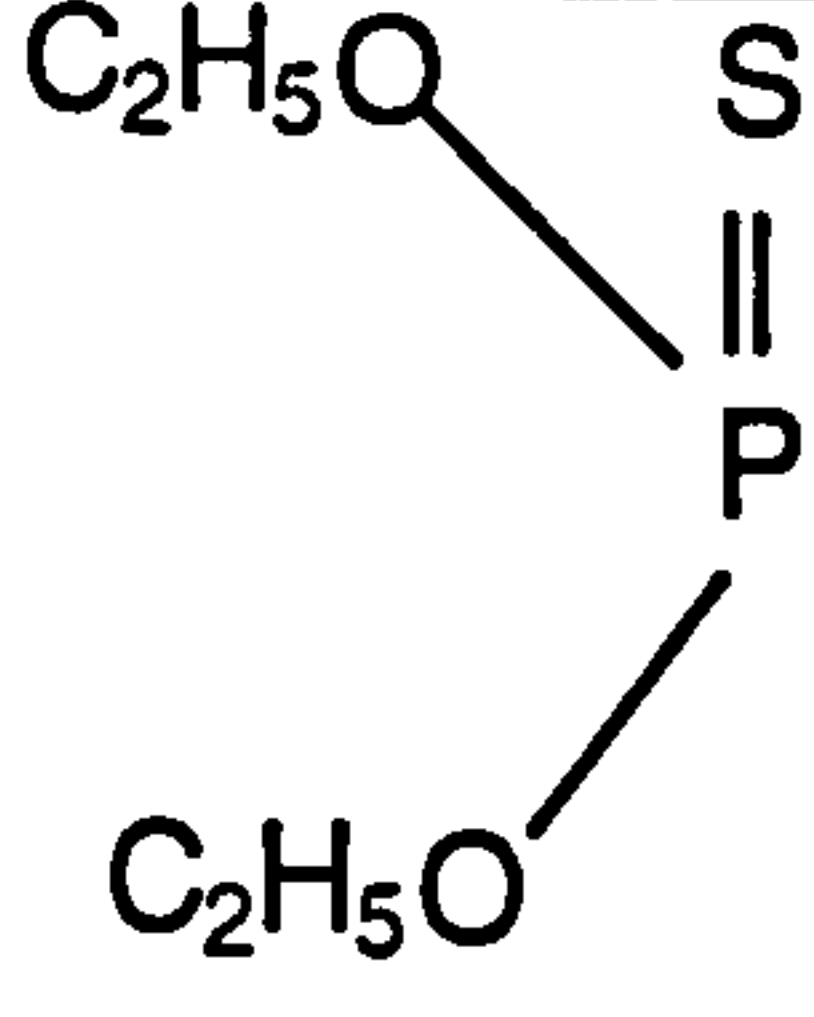
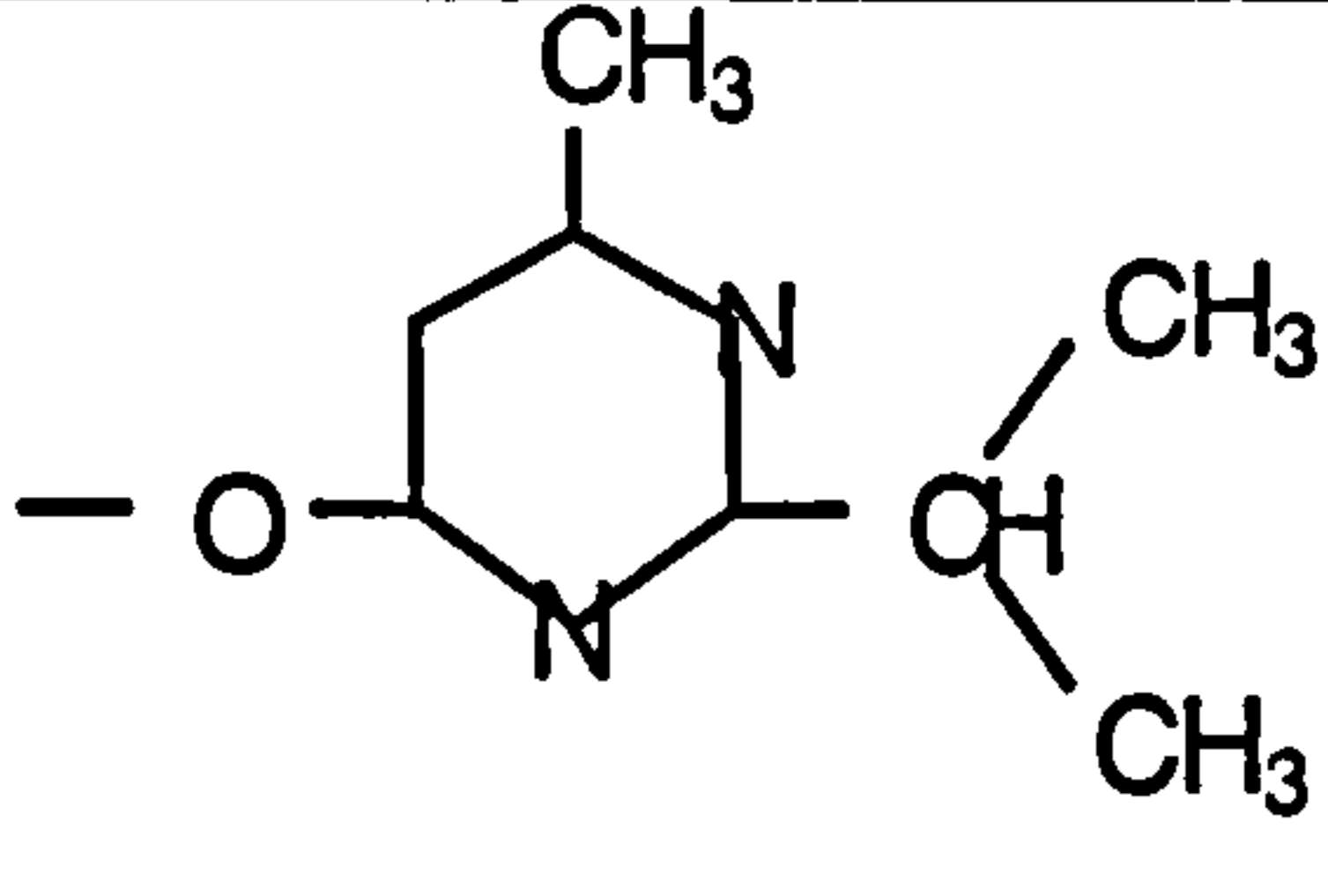
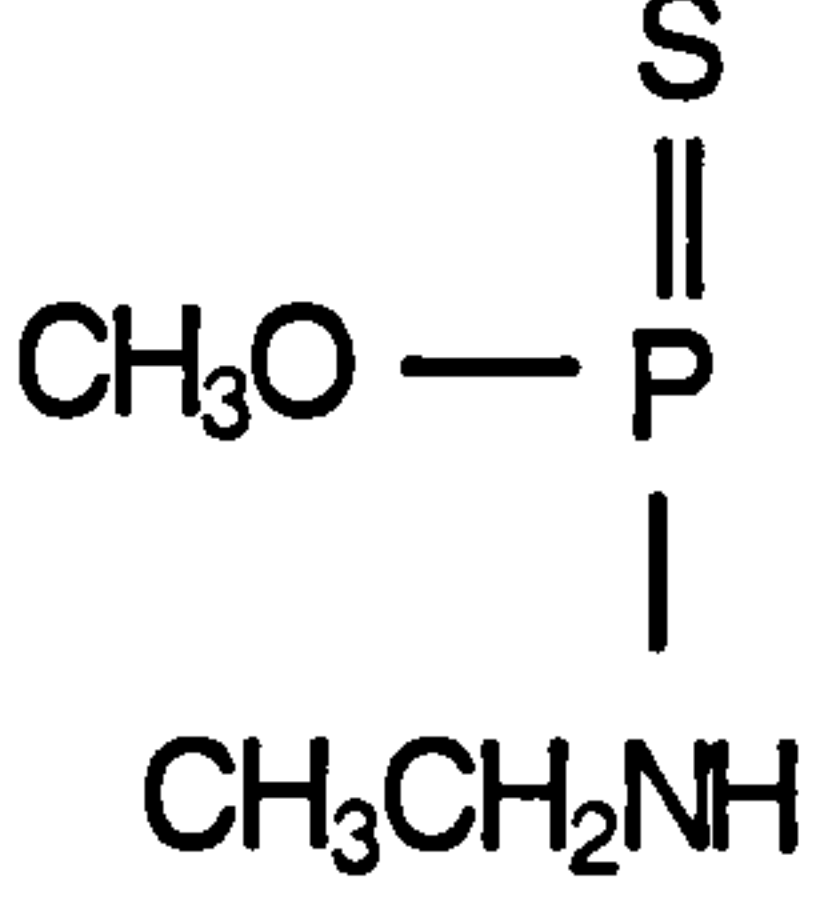
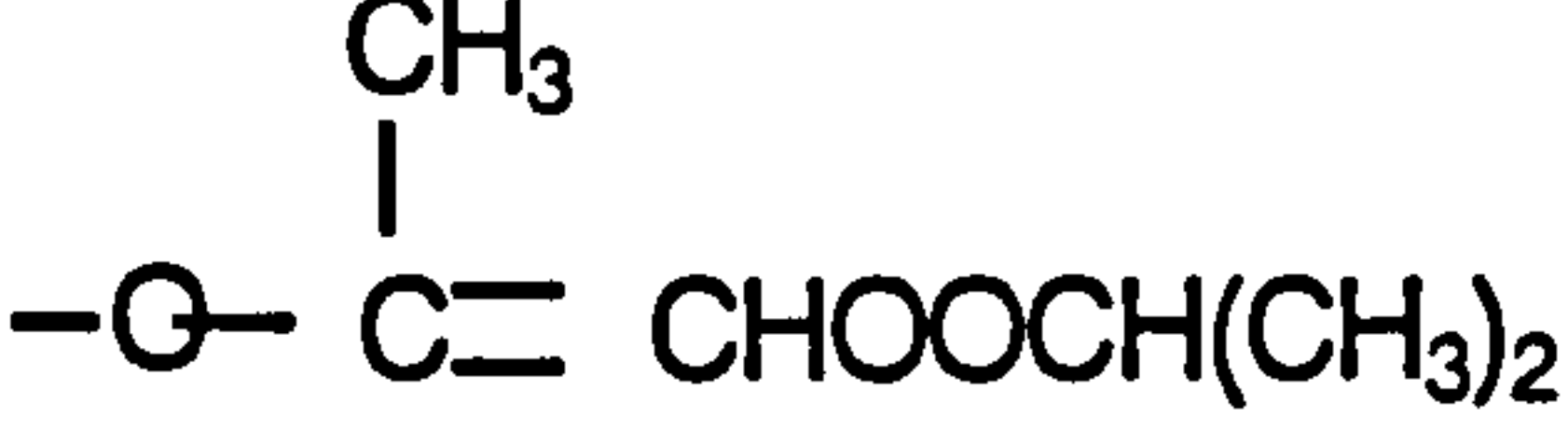
in Germany. Organophosphates have two distinctive features. First, they are generally much more toxic to vertebrates than are the organochlorine insecticides, and, secondly, they are non-persistent. It is this latter quality which brought them into agricultural use as substitutes for the organochlorines. The chemically unstable organophosphate insecticides have virtually replaced the persistent organochlorine compounds, especially with regard to use around the home and garden. However, while there has been a considerable increase in the annual use of organophosphate insecticides for crop protection since 1970, the overall increase has been less since the early 1980's although many new uses and formulations have been introduced. Phosphorothioate compounds, eg parathion, chlorpyrifos, form a group of pesticides which are generally less acutely toxic to man since they require metabolic activation to the oxon in order to exert their anti-cholinesterase effects. Table 1.1 gives a list of several phosphorothioate pesticides used occupationally.

Organophosphates exert their acute toxic action by inhibiting acetylcholinesterase ("anti-cholinesterase") in neural tissue and at the neuromuscular junction, which results in the accumulation of acetylcholine. This action, together with their chronic effects in man, is discussed more fully in chapter 2. In insects, the effects of organophosphates and carbamates are primarily those of poisoning of the central nervous system, since the insect neuromuscular junction is not cholinergic, as in mammals.

1.3 Chemical structure and classification of organophosphates

The general chemical structure of organophosphates is shown in figure 1.1. The pentavalent phosphorus has a double bond attached to either a

Table 1.1 Several phosphorothioate pesticides used occupationally.

Phosphorothioate	(R ₁ and R ₂) P	X
Chlorpyrifos		
Diazinon		
Propetamphos		

Chlorpyrifos is
O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate

Diazinon is
O,O-diethyl O-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate

Propetamphos is
2- Butenoic acid, 3((ethylamino) methoxyphosphinothioyl)oxy)-isopropyl ester

sulphur or an oxygen. Only P=O compounds (oxons), formed by metabolic oxidative desulfuration of parent P=S esters (phosphorothioates) are active as inhibitors of esterases because of a sufficiently electropositive centre at the phosphorus atom. The acidic X group, for which a wide range of chemical structures is possible, is also attached to the phosphorus. This group leaves when the phosphorus moiety undergoes nucleophilic attack by the activated serine hydroxyl group of esterases. The R groups might also be chemically very different and be attached to the phosphorus either directly (phosphinates), or through an oxygen (phosphates), or a nitrogen (phosphoramidates). When one of such groups is attached directly to the phosphorus and the other through an oxygen, the compound is called a phosphonate.

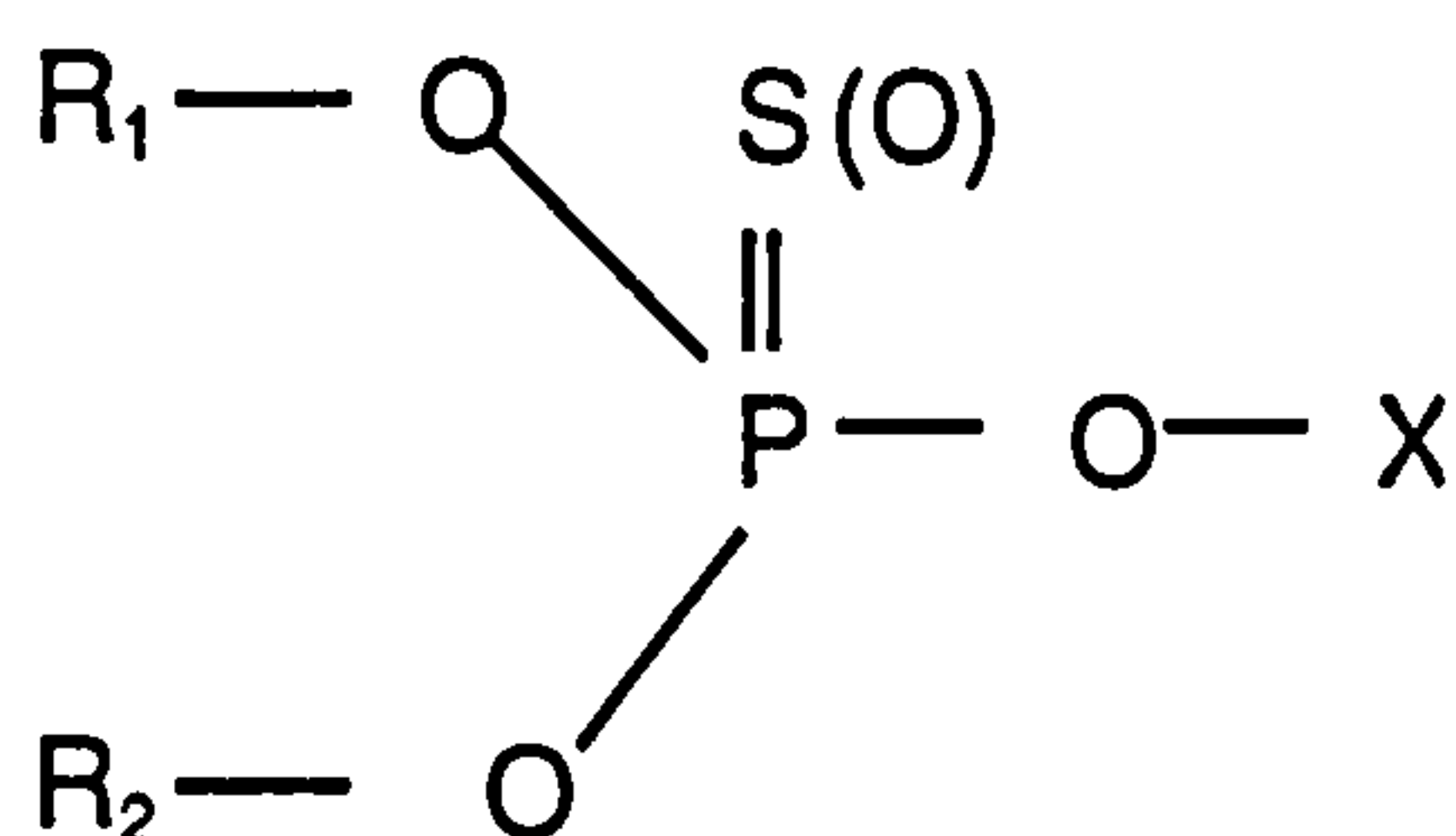


Figure 1.1

The general chemical structure of phosphorothioates (and their oxons). Oxygen atoms bound to R₁ and R₂ may be replaced with nitrogen. The R groups and the X leaving group can be chemically different.

1.4 Parathion

Parathion is O, O-diethyl-O-(*p*-nitrophenyl) phosphorothioate. It is a broad spectrum, phosphorothioate pesticide used to control many insects and mites. It has non-systemic, contact and fumigant actions and has a wide range of applications on many crops, against numerous insect species. Its structure is as follows (figure 1.2):-

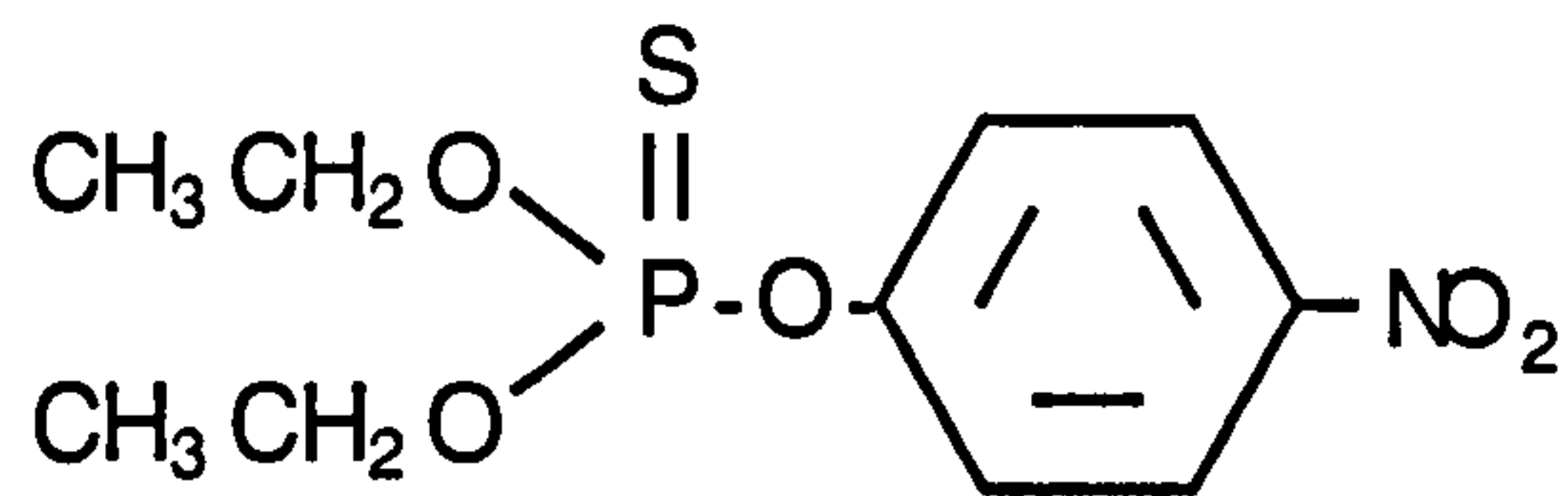


Figure 1.2 The structure of parathion.

Parathion may also be called ethyl parathion. Proprietary names include Alleron[®], Aphonite[®], Bladan[®], Folidol[®], Fosferno[®], Niram[®], Paraphos[®] and Rhodiatox[®]. The CAS registry number is 56-38-2.

Physical and chemical properties

Parathion has the empirical formula C₁₀H₁₄NO₅PS and a molecular weight of 291.3. The pure material is a yellowish liquid at temperatures above 6°C. Parathion is freely soluble in alcohols, esters, ethers, ketones and aromatic hydrocarbons, but is practically insoluble in water (20ppm) or in petroleum ether, kerosene or spray oil. Parathion is stable at a pH below 7.5.

History, formulations and uses

Parathion was developed by Schrader in 1944 and has since become one of the most widely used pesticides in the world. However, because of its relatively high mammalian toxicity, its use is now prohibited in some countries in favour of pesticides with higher selective toxicities towards insects. Parathion is banned for use as a pesticide in the UK but is still available in developing countries.

Parathion currently is used as dilute sprays, which are prepared by the operator from 15% or 25% wettable powders, or from emulsifiable concentrates of 50% or less. Dusts (5%, w/w) are used also. Aerosol

formulations containing up to 10% parathion may be used in greenhouses. Cords impregnated with parathion for fly control contain about 100mg/ linear foot. Parathion finds almost its entire use in agriculture including nurseries and greenhouses.

Toxicity

Because of its high acute toxicity, parathion is classified as a Restricted Use Pesticide (RUP). RUPs may be purchased and used only by certified applicators. The acute oral toxicity (LD₅₀) of parathion varies from about 5 to 30mg/ kg in experimental animals, depending on species and vehicle, and is extremely toxic to birds (LD₅₀ is 2.1 mg/kg in ducks).

Signs of acute poisoning by parathion are typical of exposure to organophosphorus anti-cholinesterases, however, delayed neurotoxicity has not been recognised for this compound (Johnson, 1975). Parathion is acutely toxic by all routes of exposure and human fatalities have been caused by ingestion, dermal adsorption, and inhalation (Hayes and Laws, 1990). As with all organophosphates, parathion is readily absorbed through the skin (Meister, 1987).

Chapter 2

Chapter 2 The mode of action of organophosphates

2.1 Introduction

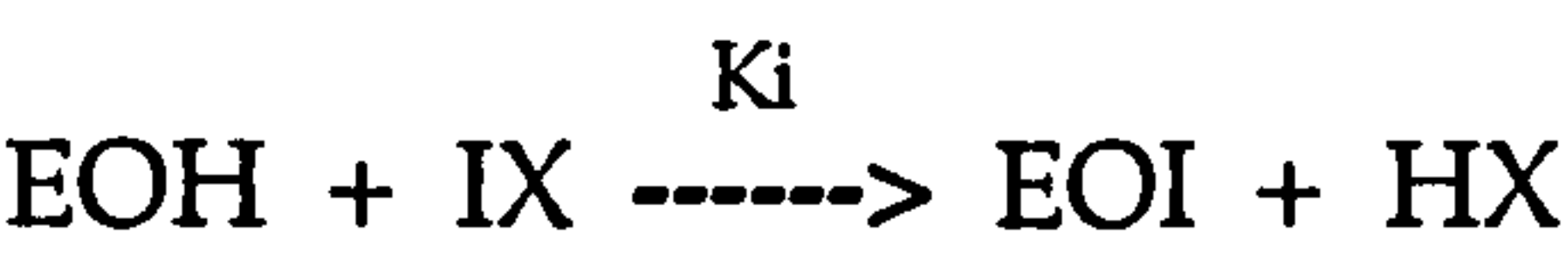
The primary toxic signs and symptoms that characterise poisoning by organophosphate compounds are acute and are well understood. They are caused by inhibition of acetylcholinesterase by phosphorylation, and accumulation of acetylcholine at susceptible receptors. Organophosphate-induced delayed neuropathy (OPIDN) also occurs in man and experimental animals following intoxication by some organophosphate esters (Lotti *et al*, 1986; Bidstrup, Bonnel and Beckett, 1953), although organophosphates with the potential to cause delayed neuropathy are not necessarily powerful cholinesterase inhibitors. Improvements in the therapy of acute poisoning mean that higher doses of some organophosphate can now be tolerated without fatal consequences, however, many organophosphates that might theoretically cause neuropathy, would only do so at a dose far above the lethal dose.

2.2 Acute toxic effects of organophosphates

The acute toxic effects of organophosphates between species will be influenced by their route of entry into the body, the level of exposure, the metabolism (activation and detoxification) of the compound and the kinetics and stability of the inhibited acetylcholinesterase complex.

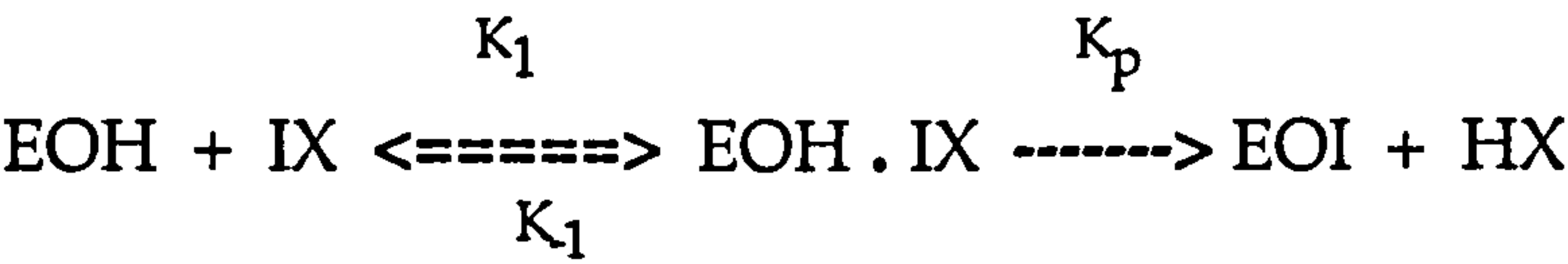
2.2.1 Kinetics of acetylcholinesterase inhibition

The inhibitory power of a compound is usually expressed as IC50, the concentration of the inhibitor giving 50% inhibition under defined conditions, or as the bimolecular rate constant (Ki), for the reaction:



where EOH represents the active enzyme and IX the inhibitor, with the leaving group X.

Aldridge (1950) suggested, for inhibition by an organophosphate, that the inhibition proceeds in two steps, with the formation of a reversible Michaelis complex preceding the next step.



The inhibition can therefore be characterised by two parameters, the affinity equilibrium constant, Ka, which is equal to K-1/K1 and the phosphorylation constant, Kp. The ratio between these two constants (Kp/Ka) gives an overall rate of inhibition (Ki), which is known as the bimolecular inhibition rate constant,

$$\text{K}_i = \frac{\text{K}_p}{\text{K}_a}$$

Two active sites are recognised in the acetylcholinesterase active centre: an anionic site and an esteratic site. The nitrogen atom and methyl groups of the choline moiety of acetylcholine interact with the anionic site, while the carbonyl atom of the acetate moiety of acetylcholine binds to the esteratic site (Koelle and Gilman, 1963). Most organophosphates lack a positive charge in the acidic group and react with the esteratic site on the acetylcholinesterase molecule, but not with the anionic site. No matter whether the anionic site is involved or not, the splitting off of the acidic group of the organophosphate compound is analogous to the splitting off of choline from acetylcholine. In contrast, the rate at which these two reactions occur are very different. The breaking of the phosphorus-enzyme bond takes from an hour to weeks, depending on the compound, whilst acetylcholine is turned over in a few microseconds (Wilson, 1951). The phosphorylation of acetylcholinesterase with paraoxon is shown diagrammatically in figure 2.1.

The inhibited acetylcholinesterase enzyme is usually stable, resulting in relatively slow recovery from intoxication. Spontaneous reactivation of the inhibited enzyme may occur and this is known to depend on the animal species and tissue, as well as on the chemical group attached to the enzyme.

The time course for acetylcholinesterase recovery at target tissues is of great importance in determining the clinical features of poisoning and differs greatly between compounds. Figure 2.2 shows this process schematically. For instance, in acute poisoning with dichlorvos (O,O,-dimethyl-O-2,2-dichlorovinyl phosphate) there is rapid spontaneous enzyme reactivation, with a half-life of 1-2 hours, and a consequent improvement clinically. However, with many organophosphates an

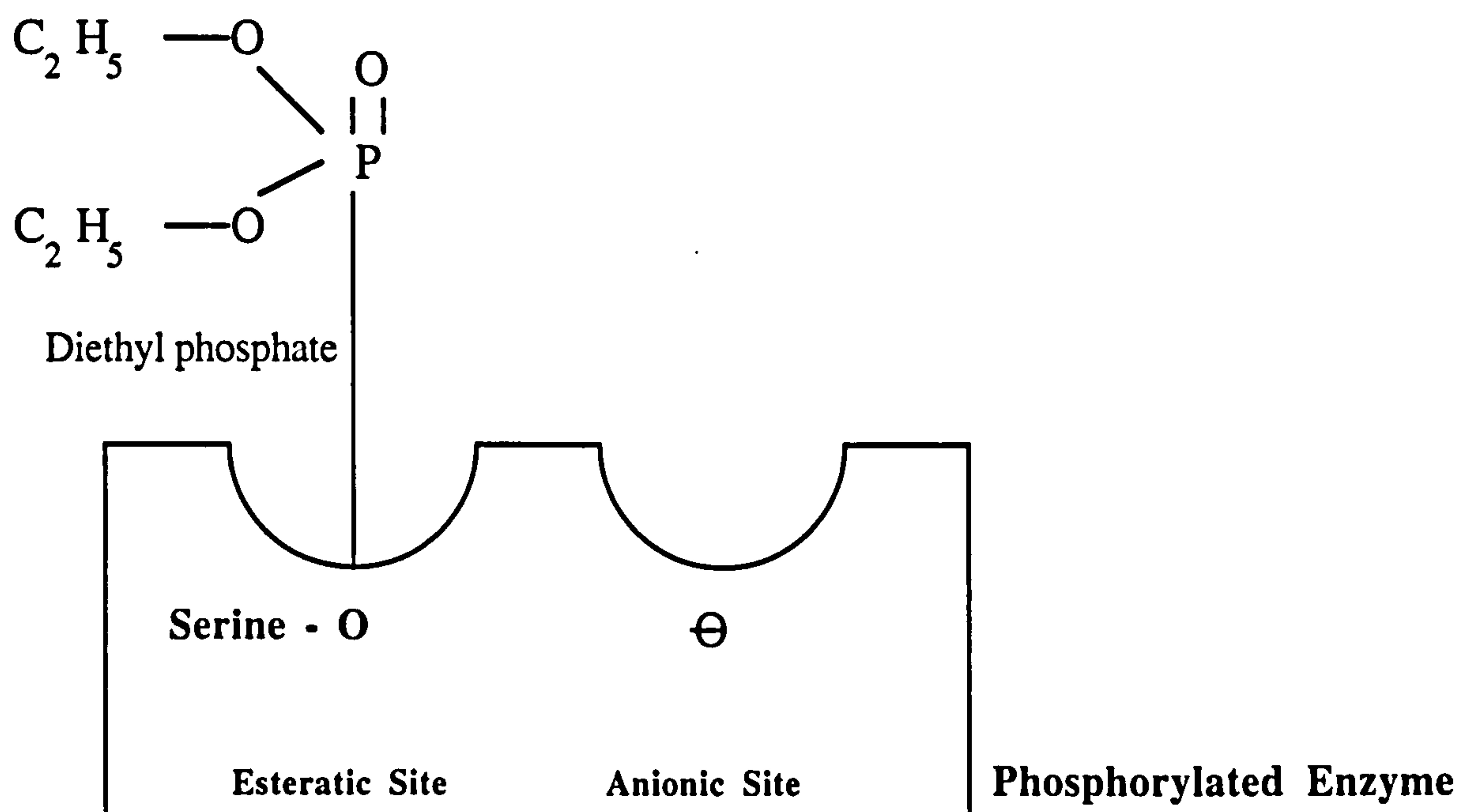
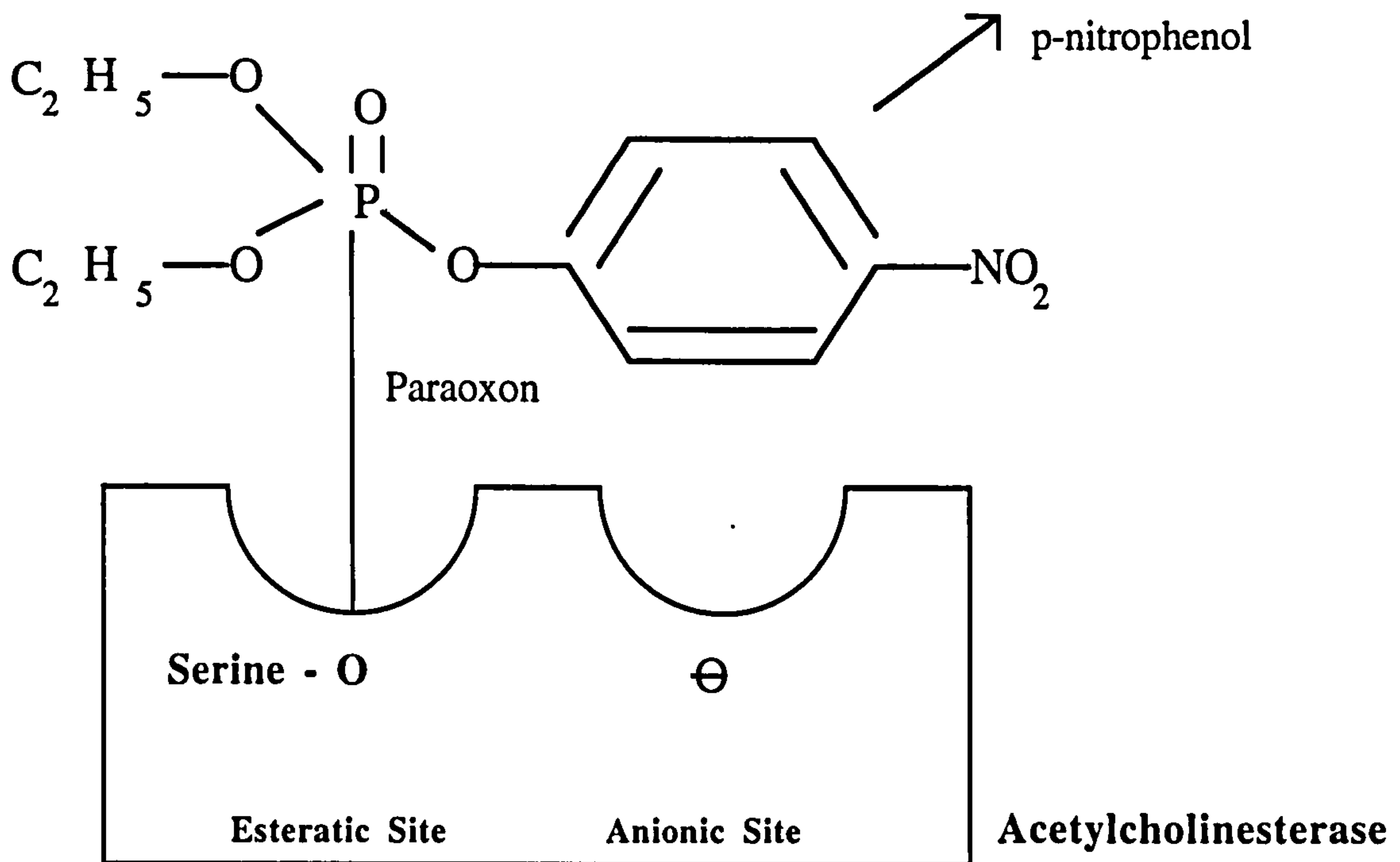


Figure 2.1

Reaction of paraoxon with acetylcholinesterase resulting in phosphorylated enzyme.

irreversibly inhibited enzyme is formed. In such instances the symptoms of intoxication are prolonged and persistent and require medical intervention, including reactivation of the enzyme with specific chemical antidotes (oximes).

2.2.2 Clinical features of acute toxicity

In man, acetylcholine is present at the terminal endings of (a) the central nervous system (b) postganglionic parasympathetic nerves, and a few sympathetic nerves such as the sweat glands (muscarinic receptors) (c) neuromuscular junctions (nicotinic receptors) (d) the autonomic nervous system at ganglionic synapses (nicotinic receptors). Inhibition of acetylcholinesterase in the nervous system results in accumulation of the neurotransmitter acetylcholine at synapses and neuromuscular junctions and excessive nervous system stimulation. This may lead to death in severe cases. A summary of acute toxicity features can be seen in table 2.1.

Death is usually caused by respiratory failure and consequent anoxia, but may be cardiovascular in origin. Four factors may contribute to respiratory failure (a) excessive secretion of the respiratory tract (b) bronchoconstriction (c) weakness of the muscles of respiration (d) failure of the respiratory centre in the brain. The relative importance of these factors depends mainly on the compound, species, route of administration, and the passage of the compound into the brain via the blood-brain barrier.

Table 2.1 A summary of acute toxicity features from organophosphate poisoning.

<p>Muscarinic features</p> <p>Increased bronchial secretion, bronchoconstriction, cyanosis, pulmonary oedema.</p> <p>Excessive sweating, salivation and lacrimation.</p> <p>Nausea, vomiting, diarrhoea, abdominal cramp.</p> <p>Urinary and faecal incontinence.</p> <p>Bradycardia, hypotension, heart attack.</p> <p>Miosis, blurred vision.</p>
<p>Nicotinic features</p> <p>Muscle fasciculation (including diaphragm and respiratory muscles).</p> <p>Generalised weakness.</p> <p>Tachycardia, hypertension.</p> <p>Pallor.</p>
<p>Central nervous system (nicotinic) features</p> <p>Confusion, headache, restlessness, anxiety, poor concentration, tremor, ataxia.</p> <p>Hypotension.</p> <p>Respiratory depression.</p> <p>Convulsions and coma.</p>

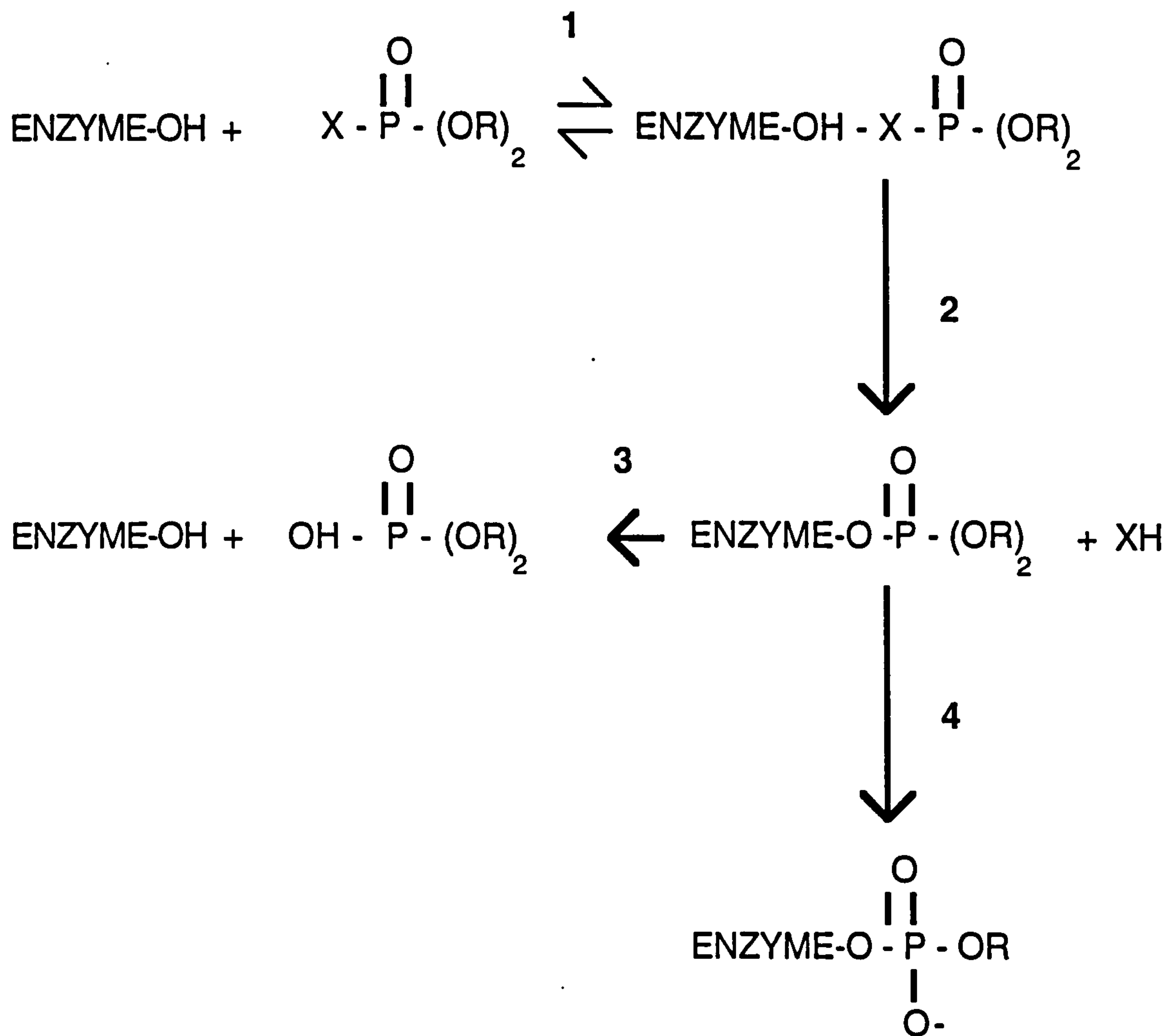


Figure 2.2

Inhibition of an esterase system by organophosphate compounds.

1 = Reversible formation of Michaelis enzyme-substrate complex.

2 = Phosphorylation of the enzyme with the loss of the leaving group (X).

3 = Reactivation of the enzyme. May occur spontaneously or on addition of oximes.

4 = 'Aging' of the enzyme on cleavage of an alkyl group.

2.2.3 Biological monitoring for acute exposure to organophosphates

Inhibition of acetylcholinesterase in the nervous system and neuromuscular junctions is paralleled by inhibition of a similar enzyme on the red blood cell and of plasma (or serum) cholinesterase (Mason and Lewis, 1989). It is generally accepted that monitoring for inhibition of the peripheral enzymes red blood cell acetylcholinesterase and plasma cholinesterase is a useful marker for organophosphate exposure. Both of these enzymes are recommended for the monitoring of occupational exposure to organophosphates (WHO report 513, 1973), but if only one test is performed, determination of red blood cell acetylcholinesterase is preferable. Acetylcholinesterase is found in nervous system tissues and therefore is considered a better indicator of biological effect than plasma cholinesterase.

It is possible to estimate absorption of organophosphates by analysis for their metabolic products in urine several days after exposure and in association with lesser exposures than those necessary to produce cholinesterase inhibition (Knaak *et al*, 1979). This will give information on recent exposure, whilst measurement of enzyme levels, especially red blood cell acetylcholinesterase values, gives the physiological effect of exposure during the preceding month or so. Both kinds of information may be of value.

Health and Safety Executive guideline MS 17 (1987) suggests that workers should be medically examined if plasma cholinesterase activity has been shown to have fallen by more than 30% of pre-exposure levels during routine monitoring. The medical officer may then at his own discretion,

having considered the nature of the work involved and the clinical symptoms (if any), recommend that the worker be suspended from further exposure to organophosphates until considered fit to resume further work. A recommendation to resume normal work should be based on both clinical evidence and the results of biological monitoring.

2.3 Delayed neurotoxic effects of organophosphates

Another syndrome, that of organophosphate-induced delayed neuropathy (OPIDN), is caused by some phosphate, phosphonate and phosphoramidate esters, only a few of which have ever been used as insecticides. Regardless of the severity of anti-cholinesterase effects, there is a delay after intoxication before neuropathic signs and symptoms appear. Symptoms of OPIDN are well documented in humans following exposure to organophosphates such as chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-pyridyl) (Lotti *et al*, 1986), methamidophos (O,S-dimethyl phosphoroamidothioate) (Senanayake and Johnson, 1982), mipafox (Bidstrup *et al*, 1953) and TOCP (tri-ortho-cresyl phosphate) (Inoue *et al*, 1988), amongst others. High inhibition of lymphocyte neuropathy target esterase predicted the development of OPIDN when measured soon after massive poisoning with chlorpyrifos, but several days before clinical signs were apparent (Lotti *et al*, 1986). This suggested a means for monitoring exposure to neurotoxic organophosphates. However, unlike in the hen, the threshold for neuropathy target esterase inhibition in man has yet to be determined. This indicates care when extrapolating between the species.

A complete account of all organophosphates tested for neurotoxic potential has been given by Johnson in three reviews (Johnson 1975a;

1975b; 1982). A list of organophosphorus compounds shown to produce OPIDN in hens is given in table 2.2.

Parathion is not believed to cause OPIDN and therefore this syndrome will be discussed no further (Johnson, 1988).

2.4 Long term effects of organophosphates

The classical picture of acute anti-cholinesterase insecticide intoxication, which was first described by DuBois (1948), has become more complicated in recent years by the recognition of additional and persistent signs of toxicity not previously associated with these chemicals. Recent studies have suggested that long term neurological effects may result from a single acute episode of poisoning or chronic low level exposure. Studies carried out by Ecobichon (1982) have shown that effects involving neurobehavioral, cognitive and neuromuscular functions frequently persist for several months following single exposure to high concentrations of organophosphates. A study of sixteen pesticide workers exposed primarily to organophosphate insecticides for 10-15 years reported a wide range of persistent signs of toxicity (Ecobichon, 1982). The symptoms included tinnitus, pyrexia, ataxia, tremor, paraesthesia, paralysis, loss of memory, lassitude, generalised weakness, mental confusion, restlessness, anxiety and depression.

In an epidemiological study of long-term effects of organophosphates (Savage *et al*, 1988), 100 pairs of individuals with a previous history of poisoning were compared to matched controls. Significant differences were not found in blood chemistry or EEG, but were found in psychometric analysis and a test of motor reflexes.

Table 2.2

Organophosphorus compounds shown to produce OPIDN in hens.

Compound	Route	Lowest lethal dose (mg/ kg)	Lowest neurotoxic dose (mg/ kg)
DFP	s.c.	>1	1
Mipafox	s.c.	>20	20
Chlorpyrifos	s.c.	200	200
Coumaphos	s.c.	100	100
Disulfoton	s.c.	>50	32
Phorate	s.c.	48	32
DEF	i.p.	100	50 x 5
Merphos	s.c.	600	600
TOCP	p.o.	200	100ppm
Cyanofenphos	p.o	>10	10
EPN	s.c.	80	40
Desbromleptophos	p.o.	>50	50

- DFP is O,O-diisopropyl fluorophosphate
- DEF is S,S,S-tributyl phosphorotrithioate
- TOCP is tri-*o*-cresyl phosphate
- EPN is O-ethyl O-4-nitrophenyl phenylphosphonothioate

Rosenstock and co-workers (1991) carried out a retrospective study of agricultural workers who had experienced a single episode of poisoning with organophosphates on average two years earlier. The poisoned group performed worse at neuropsychological testing and assessments of visual attention, visual memory, visuomotor speed, sequencing and problem solving, motor steadiness and dexterity compared to matched controls.

Other reports have suggested that long-term effects on the central and peripheral nervous systems may also be associated with relatively low level organophosphate exposure. These effects range from neurobehavioural and EEG abnormalities to electrophysiological changes in animal diaphragmatic and skeletal muscles (Kelly, 1990). Other studies have shown psychological, behavioural or electrophysiological changes after exposure of humans to low doses of organophosphates. For example, Stephens *et al* (1995) found neurobehavioural changes in a group of farmers who used organophosphate sheep-dips compared to a control group of quarry workers.

The National Farmers Union report acute and long-term toxic effects associated with the use of organophosphates present in sheep-dips for the control of scab, a mite which lives in the skin of this animal. Studies of such workers are difficult to evaluate since clinical symptoms are often non-specific in nature and estimations of toxicity are limited by the sensitivity of the various analytical, biochemical, neuropsychological and neurophysiological tests used.

A further distinct manifestation of exposure to several organophosphate compounds has recently been described by clinicians in Sri Lanka involved in the treatment of suicide attempts (Senanayake and

Karalliedde, 1987). This paralytic condition, called the “intermediate syndrome” consisted of a sequence of neurologic signs that appeared some 24 to 96 hours after the acute cholinergic crisis but before the expected onset of delayed neuropathy. The major effects were muscle weakness, primarily affecting muscles innervated by the cranial nerves but some limb involvement was also apparent. There was a distinct risk of death during this time interval because of respiratory depression and distress which required urgent ventilatory support. There were no obvious clinical differences during the acute intoxication phase between those patients who developed the “intermediate syndrome” and others who did not.

Chapter 3

Chapter 3 Xenobiotic metabolism

3.1. Introduction

Man is exposed to a wide variety of chemicals daily in food, at work and in the general environment. Many exogenous compounds (xenobiotics) such as drugs are taken into the body deliberately, but many environmental compounds cannot be avoided and are absorbed into the body through the skin, inhaled, or swallowed unintentionally (figure 3.1).

Xenobiotic metabolism can be divided into two phases: phase 1 introduces or exposes a functional group in the molecule which can then be conjugated in phase 2. The role of metabolism, therefore, is to introduce polarity to otherwise lipophilic xenobiotics to enable their effective excretion.

Table 3.1 Reactions classed as phase 1 or phase 2 metabolism

Phase 1	Phase 2
Oxidation involving cyt. P450	Glucuronidation
Other oxidation reactions	Sulphation
Reduction	Methylation
Hydrolysis	Acetylation
Hydration	Amino acid conjugation
Isomerisation	Glutathione conjugation
Dethioacetylation	Fatty acid conjugation
	Condensation

Most xenobiotics are metabolised by a variety of enzymes, and these metabolic processes generally produce metabolites that are less toxic than the parent compound. However, this is not always the case and some processes give rise to metabolites that are more reactive, eg the oxidative

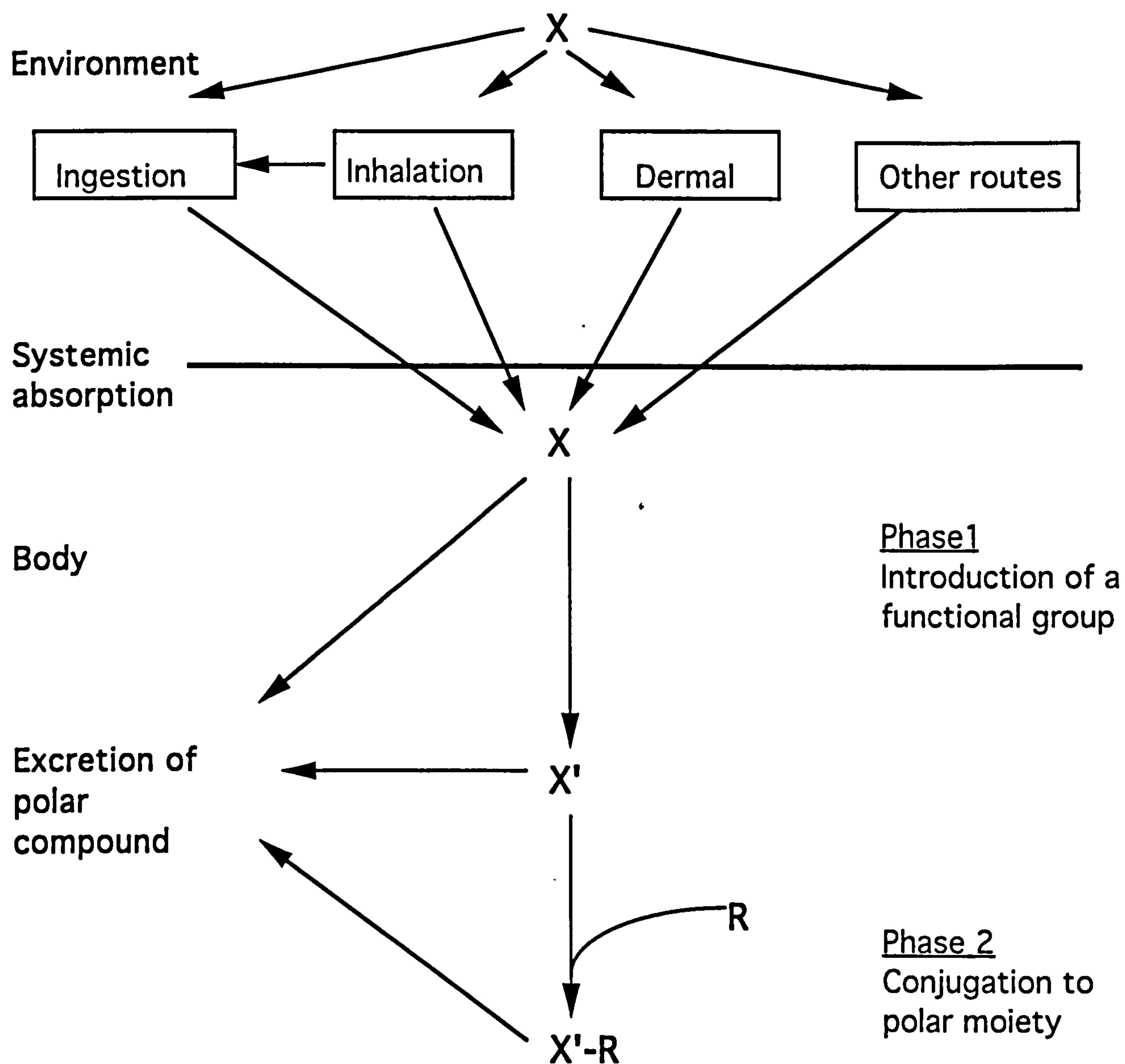


Figure 3.1
The central role of metabolism in the excretion of metabolites

desulphuration of phosphorothioates by phase 1 reaction(s) produces highly toxic oxons, causing toxic effects. Metabolic profiling is therefore necessary to assess the effect and toxicity of xenobiotics, and to indicate individual susceptibility to any toxic effects.

Phase 1 enzymes potentially involved in the metabolism of phosphorothioates include the cytochromes P450, esterases and flavin monooxygenases. In terms of phase 2 enzymes, it has been suggested that glutathione-S-transferases, glucuronyl-S-transferases, sulphotransferases and epoxide hydrolase are major pathways in the detoxification of exogenous and endogenous compounds (Jakoby and Habig, 1980).

In this chapter a general review of xenobiotic metabolism is presented concentrating on aspects pertinent to this thesis.

3.2 The cytochrome P450 system

The cytochromes P450 (EC 1.14.14.1) comprise a family of b-type haemoproteins with an identical prosthetic group (a thiolate-bound haem) and mechanism of catalysis (activation of oxygen without activating the substrates), but widely different apoprotein structures which are responsible for their different substrate specificities. The general catalysed reaction of the cytochromes P450 can be summarised as follows:



where R is the substrate

Liver microsomes from all mammalian species contain numerous P450 enzymes, each with the potential to catalyse numerous xenobiotic

transformation reactions, such as hydroxylation, epoxidation, dealkylation, desulphuration, oxygenation and dehydrogenation. The broad and often overlapping substrate specificity of P450 isoforms precludes the possibility of naming these enzymes for the reactions they catalyse.

Cytochrome P450 evolution is believed to have begun 2-3 billion years ago and many of the xenobiotic-metabolising enzymes evolved due to a need to metabolise plant toxins and similar compounds (Gonzalez and Nebert 1990). One consequence of human P450 gene evolution is the polymorphism of drug metabolism, leading to marked differences in the response of individuals to the toxic and carcinogenic effects of drugs and other environmental chemicals. The differences between human individuals, races or ethnic groups, are either at the level of expression of some forms (eg CYP1A1 after induction), or at the presence or absence of a particular form (eg CYP2D6, CYP3A5), and may become clinically manifest in quantitative differences in the metabolism of certain drugs resulting in "poor" or "extensive" metabolisers (Gonzalez *et al*, 1991).

The amino acid sequence of numerous P450 enzymes has been determined, largely by recombinant DNA techniques, and such sequences now form the basis for classifying and naming P450 enzymes (Nelson *et al*, 1996). In general, P450 enzymes with less than 40% amino acid sequence identity are assigned to different gene families (gene families 1, 2, 3, 4 etc). P450 enzymes that are 40-50% identical are assigned to different subfamilies (eg 2A, 2B, 2C, 2D, 2E). P450 enzymes that are more than 55% identical are classified as members of the same subfamily (eg 2A1, 2A2, 2A3).

The liver microsomal P450 enzymes involved in xenobiotic biotransformation belong to three main P450 gene families of both humans and rodents, namely, CYP1, CYP2 and CYP3. Liver microsomes also contain P450 enzymes encoded by the CYP4 gene family, substrates for which include several fatty acids, but relatively few xenobiotics. The liver microsomal P450 enzymes in each of these gene families generally belong to a single subfamily (ie CYP1A, CYP3A and CYP4A). A notable exception is the CYP2 gene family, which comprises five subfamilies (ie CYP2A, CYP2B, CYP2C, CYP2D and CYP2E). The number of P450 enzymes in each subfamily differs from one species to the next.

Man, in contrast to experimental animals, shows large interindividual variations in P450-catalysed biotransformations, due to the many factors discussed in the next few chapters. Difficulties often arise when extrapolating animal data to man because P450 isoforms are not conserved between species and differences are known to occur in catalytic and regulatory specificities between human P450 isoforms and their rat orthologues (table 3.2). Differences in the levels of individual P450 isoforms and indeed the expression of distinct isoforms may lead to differences in the metabolism of alleged probe substrates between species, and may also influence the selectivity of inhibitor probes.

The major human P450 subfamilies are CYP3A and CYP2C, which account for about 30% and 20% of the total P450 pool based on immunochemically detectable proteins, with CYPs 1A2, 2E1, 2A6, 2D6 and 2B6 comprising 13%, 7%, 4%, 2% and <1%, respectively (Shimada *et al*, 1994). Levels of P450 isoforms in male rats are very different with CYP2C11 accounting for 54% of the total P450 content, CYP3A2 being 17% abundant and CYP1A2 being

expressed at much lower levels (2%) in untreated rat liver (Nedelcheva and Gut, 1994).

Table 3.2 Sequential homology between rat and human P450 forms

P450 form		Sequential homology ^a (%)
Rat	Human orthologue	
CYP1A1	CYP1A1	80 (78)
CYP1A2	CYP1A2	75 (70)
CYP2A3	CYP2A6	(85)
CYP2B1	CYP2B6	78 (74)
CYP2B2	--	-- ^b
CYP2C6	CYP2C10*	(75)
CYP2C11	CYP2C9	80 (77)
CYP2C13	CYP2C8	74 (68)
CYP2D1	CYP2D6	(71)
CYP2E1	CYP2E1	75 (78)
CYP3A1	CYP3A4	(78)
CYP3A2	CYP3A5	(71)
CYP4A1	CYP4A9	-- ^b

^a Similarity of cDNA and amino acid (in parenthesis) sequence stated

^b No data available regarding existence of orthologous form

* Discontinued number; existence in doubt

(Nelson *et al* (1996). Pharmacogenetics 6: 1-42)

Table compiled by Soucek and Gut (1992). Xenobiotica 22: 83-103.

3.3 The flavin monooxygenase system

The action of the flavin monooxygenases (FMO, EC 1.14.13.8) constitutes an important means of terminating the pharmacological and toxicological action of a wide number of xenobiotics, including some organophosphate pesticides. Like the cytochromes P450, FMO are located in the endoplasmic reticulum of mammalian cells. It is believed that FMO evolved to detoxify

nucleophilic heteroatom-containing chemicals, or their metabolites, present in plants that would otherwise inactivate or be metabolised by the cytochromes P450, or covalently modify critical tissue macromolecules.

The FMO catalyse the *N*-oxygenation of tertiary amines, and also the oxygenation of organosulphur-, selenium-, phosphorus- and other heteroatom-containing chemicals that are likewise more efficiently excreted after oxygenation. In contrast to the cytochromes P450, FMO generally converts heteroatom-containing compounds to products with decreased potential for toxic or carcinogenic properties. Cytochromes P450 can oxidise nucleophilic heteroatoms to heteroatom oxides that, in some cases, are the same as the products from FMO, but the P450s do so considerably less efficiently than FMO.

The mechanism of action of FMO involves reaction of the FAD prosthetic group, firstly with NADPH and then molecular oxygen. These initial reactions occur in the absence of substrate and the energy for catalysis is therefore present before any contact with the xenobiotic. Organophosphate pesticides known to be substrates for the FMO include phorate (O, O,-diethyl-S-(ethylthiomethyl) phosphodithioate) and disulfoton (O, O,-diethyl-S-(ethylmercaptoethyl) phosphodithioate) (Tynes and Hodgson, 1985).

There are five distinct FMO gene families encoding five forms of mammalian FMO, but the most functionally prominent form of the enzyme in adult human liver, FMO3, does not appear to be expressed to a great extent in adult rat liver. Little is known about the factors that control FMO regulation, but hormone and dietary factors appear to largely

regulate its expression. This is, however, species and tissue specific and probably occurs independently of other factors.

3.4 The esterases

Depending on their interaction with organophosphates, the esterases are loosely divided into three classes: The first hydrolyse organophosphates and consequently play an important detoxification role while a second group are inhibited by them; another small group do not react with organophosphates compounds and are of no interest in connection with these pesticides. Interindividual variations in some of the esterases involved in organophosphate toxicity, notably the detoxification enzymes, could be expected to influence susceptibility to toxic effects.

A simple, clear classification of esterases is extremely difficult to set out since many have overlapping substrate specificity and will hydrolyse more than one substrate, albeit to varying degrees. Esters, amides, hydrazides and carbamates can be hydrolysed by various esterase enzymes present in body tissues and fluids. The hydrolysis of esters is as follows:



RCOOR_1 is the hydrolysable ester substrate, RCOOH and R_1OH are the acid and alcohol products.

The classification of the esterases by the International Enzyme Commission (EC) is based on the substrate type for which these enzymes show the greatest affinity. Many substrates have been used in an attempt to further classify esterases which has resulted in a very confusing picture.

This has particularly been the case with carboxylesterases which possess a high degree of multiplicity with a large number of isoenzymes coded for by separate gene loci (Hendrich and von Diemling, 1987). Carboxylesterase isoenzymes have many common substrates and this has added to confusion over their classification.

Heymann (1989) reasoned that carboxylesterases must be present to hydrolyse endogenous compounds and attempted to simplify the literature by identifying such substrates. He proposed that these substrates alone should form the basis of a new classification system, hence eliminating the situation where the same isoenzyme had different names from different laboratories.

An additional classification introduced by Aldridge in 1953 was used mainly in toxicological literature and paralleled the EC system. It is based on the observation that paraoxon (O, O diethyl *p*-nitrophenyl phosphate), an irreversible inhibitor of serine-containing esterases, may, in fact, act as a substrate for others. Thus, paraoxon is a substrate for A-esterases, an inhibitor of B-esterases and without influence on C-esterases. C-esterases are not included in this chapter since they are not affected by organophosphate compounds.

The A-esterase “paraoxonase”, and B-esterases cholinesterase, acetylcholinesterase and carboxylesterase, will be discussed in this chapter.

3.4.1 A-esterases

A-esterases are a group of enzymes with the ability to detoxify organophosphorus anti-cholinesterase nerve gases and insecticides, and

carbamate insecticides by hydrolysis, without themselves being inhibited. The mechanism by which the A-esterases hydrolyse organophosphates is unclear. However, the complex formed between organophosphate and enzyme appears to be unstable and this is shown in a high turnover rate leading to rapid regeneration of free enzyme (Mackness 1989).

The most common substrate for studying A-esterase activity is paraoxon, which has led to the common name "paraoxonase" being used in the literature. The highest levels of activity for this enzyme have been found in the liver microsomes and plasma of man and small rodents (McCracken *et al* 1990). Paraoxonase has the ability to detoxify paraoxon by hydrolysis to *p*-nitrophenol and diethylphosphate with an apparent K_m of 0.4mM (Gil *et al* 1993).

Paraoxonase requires calcium for maximum catalytic activity and stability, although the mechanism for the interaction with calcium is unknown at this time (Vitarius and Sultatos, 1995b). Unlike the cholinesterases, paraoxonase is not inhibited by physostigmine or organophosphate compounds, and indeed, some of the latter are substrates for the enzyme. It is, however, inhibited by chelating agents such as EDTA and by the organomercurial sulfhydryl reagent, *p*-hydroxymercuribenzoate. Recent studies (Sorenson *et al* 1995) have shown that paraoxonase does not have a cysteine residue at its catalytic centre as commonly thought for many years. Sorenson suggests that inhibition of the activity of paraoxonase by *p*-hydroxymercuribenzoate may be due to steric hindrance resulting from the introduction of a large substituent near a region of the molecule critical for substrate binding, rather than the presence of a free sulfhydryl group at the enzymes' active centre.

Studies carried out by Little *et al* (1989), have shown that the A-esterases responsible for hydrolysis of organophosphates with P-F and P-CN bonds (including DFP, mipafox, soman and sarin) are poor substrates for paraoxonase. These organophosphates are hydrolysed by the esterase currently classified as organophosphorus acid anhydrase (EC 3.1.8.1, OPA) which requires Mn^{++} or Co^{++} for maximum activity. Organophosphorus acid anhydrase activity is localised almost exclusively in the soluble fraction of the liver and is distinct from paraoxonase, which is only present in the microsomes (Broomfield *et al* 1989).

Like many other esterases, no known endogenous substrates have been identified. However, the human serum enzyme is closely associated with the high density lipoprotein (HDL) complex (Kelso *et al* 1994) and there is growing evidence that it may protect against low density lipoprotein (LDL) oxidation and so prevent atheroma formation (Watson *et al* 1995) through hydrolysis of leukotriene-like compounds. Further links between HDL and paraoxonase activity have been based on observations of patients with medical conditions such as fish-eye disease and Tangier disease which are associated with disturbances in lipid levels (Mackness *et al* 1987; Dumon *et al* 1986).

Human plasma paraoxonase activity has been found to vary considerably between individuals (Eckerson *et al* 1983; Mutch *et al* 1992). The observed individual variability in the ability to detoxify the active oxons of phosphorothioate pesticides by hydrolysis may be of considerable importance in protection against the inhibition of B-esterases, such as acetylcholinesterase and neuropathy target esterase (NTE), at target tissues.

Recent studies (La Du, 1996) have shown that human serum paraoxonase (PON1) is polymorphic occurring as two forms that differ only by the amino acid at position 192, which is glutamine in type Q (formerly known as type A) and arginine in type R (formerly type B). Furlong and co-workers (Furlong *et al* 1989) determined that 48% of Caucasians are homozygous for the Q allozyme, 43% are QR heterozygotes and the remainder classified as RR homozygotes. The PON1 R variant was eight times more active towards paraoxon than the Q allozyme, although the two forms were shown to be similar in their ability to hydrolyse the aromatic acid ester phenylacetate and chlorpyrifos-oxon. The two human allozymes are presumed to be products of the *PON1* gene which has now been assigned to chromosome 7 through its linkage with the cystic fibrosis gene (Eiberg *et al* 1985). The human paraoxonase/arylesterase (PON1) enzyme has now been reclassified as aryldialkylphosphatase (EC 3.1.1.2).

A study by Davies *et al* (1996) investigated the ability of PON1 to hydrolyse diazoxon and the nerve gases sarin and soman, in addition to paraoxon. It was shown that diazoxon, sarin and soman were hydrolysed far less efficiently by type R than type Q sera, which is the opposite to that noted for paraoxon. The authors suggested that these results help to explain the large individual differences in sensitivity to organophosphate pesticides processed through the P450/PON1 pathway or hydrolysed directly by PON1. However, saturating substrate concentrations were used in order to determine the rates of hydrolysis of each organophosphate and no attempt was made to assess the situation at concentrations relevant to those experienced in the workplace or environment.

Several species of bird and fish have been shown to have low paraoxonase activity (Brealey *et al* 1980). The low serum paraoxonase activity for birds

and fish compared to mammals is believed to greatly influence the observed selective toxicity of organophosphate insecticides towards avian and fish species. Recent studies (Primo-Parmo *et al* 1996) with turkey and chicken sera have shown that these species have a *PON*-like gene with approximately 70% identity to human *PON1*. The authors suggest that the product of this gene, "PON2", if expressed in these species, would be unable to hydrolyse paraoxon. Conversely, a wide variety of mammalian species have high paraoxonase activity. For example, paraoxonase activity was 7-fold greater in rabbit serum than rat, with the dose of paraoxon required to produce similar degrees of cholinesterase inhibition and similar signs of toxicity differing by 4-fold for the two animals (Costa *et al* 1990).

In summary, fish and birds are extremely susceptible to organophosphate toxicity, whereas certain mammals, eg rabbits, that have relatively high paraoxonase activity show greater resistance to poisoning by these agents. This observation appears to hold true particularly when the exposure is low and chronic, rather than a high acute dose.

3.4.2 Cholinesterase

Cholinesterase (EC 3.1.1.8), formerly pseudocholinesterase, is found in almost all major systems of the mammalian body, although there is considerable variation in the level of activity between species (Silver, 1974). Despite its widespread presence, no biological function has been established to date, although involvement in lipid and lipoprotein metabolism has been suggested (Chu *et al*, 1978). Cholinesterase is, however, known to hydrolyse a range of drugs including suxamethonium, aspirin, diacetylmorphine, substance P, cocaine, procaine

and other local anaesthetics as well as steroid esters (Lockridge *et al*, 1980; Lockridge, 1982; Lockridge, 1990; Stewart *et al*, 1977).

In the past, gross deficiency of cholinesterase activity sometimes caused fatalities by much prolonging the effects of succinylcholine, a usually very short-acting muscle relaxant used in anaesthesia (Lockridge, 1990). One report (Prody *et al*, 1989) has suggested that individuals with a cholinesterase deficiency may be more susceptible to poisoning by certain organophosphate insecticides, but this is largely unsubstantiated.

The specificity of cholinesterase is due to the arrangement of its active centre which consists of an anionic site and a serine esteratic site. Charged N- and methyl groups of choline esters bind to the anionic sites and hydrolysis of the substrate occurs as a two stage process. Firstly, the acyl group binds to the serine residues of the esteratic site and choline is released. Next the transiently acetylated enzyme reacts with water to yield acetic acid and regenerate the enzyme (Williams, 1985).

In man, cholinesterase is synthesized in the liver and occurs in the blood plasma in four main molecular forms, designated as C1, C2, C3 and C4 according to their electrophoretic mobility on gels (Masson, 1989). Serum cholinesterase is controlled by at least four allelomorphic genes at the same locus which are autosomal and co-dominant. The alleles have been designated ChEu, ChEa, ChEf, ChEs (Whittaker, 1986). The homozygous genotype ChEu ChEu (u = usual) is found in 95% of Caucasians, whereas 4% will be ChEu ChEa (a = atypical), and 0.5% ChEu ChEs (s = silent) (Whittaker, 1986). Heterozygous atypical individuals exhibit an average cholinesterase activity corresponding to about 75% of the average activity of homozygous "usual" individuals. The average cholinesterase activity

in plasma from individuals with the genotypes ChEu ChEf (fluoride) and ChEu ChEs corresponds to about 85% and 50% respectively of homozygous "usual" individuals. The silent phenotype (genotype ChEs ChEs) occurs in about 1:100 000 Caucasians and the plasma of these individuals contains no detectable cholinesterase activity (Brock and Brock, 1990).

There is a single copy of the gene *BUChE* for butyrylcholinesterase. It is located on chromosome 3 in region 3q21-25. The first genetic variant of cholinesterase was identified in 1956, designated "atypical cholinesterase", and characterised as a high K_m variant (Kalow and Genest, 1957). Other variants were discovered later and the molecular basis of all except some extremely rare variants has been elucidated in recent years (La Du *et al*, 1992). The "atypical" variant represents the amino acid alteration 70 Asp--->Gly. The existence of an "f" variant has been recognised for several decades, but DNA data has now shown that there are actually two "f" variants (243 Thr--->Met and 390 Gly--->Val). There are a series of "silent" variants, a special case being the K variant (Bartles *et al*, 1992) (with amino acid change 539 Ala--->Thr) which reduces enzyme activity by about one-third without affecting affinities. This mutation occurs in linkage disequilibrium with the "atypical" variant, i.e. the 70 Asp--->Gly mutation tends to be accompanied by the 539 Ala--->Thr mutation. About 1% of Caucasians are homozygous for the K variant which makes this the most frequently encountered *BUChE* mutation.

The atypical forms of the enzyme can be distinguished from "normal" species by their increased resistance to inhibition by dibucaine (Kalow and Genest, 1957) and fluoride (Harris and Whittaker, 1961) using benzoylcholine as substrate. Normal serum cholinesterase is inhibited about 80% by this compound while atypical homozygotes show only about

20% inhibition. Heterozygotes exhibit an intermediate value of about 60% inhibition, that is they have a dibucaine number (DN) of 60. Determination of the DN therefore distinguishes between normal homozygotes, atypical heterozygotes and atypical homozygotes, which is not possible by assay of cholinesterase activity alone because of the overlap of activity ranges.

Depression of serum cholinesterase activity has been reported in persons with liver disease, anaemia, malignant neoplasia, pulmonary tuberculosis, acute infections and chronic debilitating diseases, and malnutrition. A nutrition survey of elderly people in Great Britain (DHSS, 1979) found that serum cholinesterase activity correlated significantly with arm circumference, skin fold thickness, protein intake and serum albumin concentration. This has been corroborated by Lepage *et al* (1985). The serum cholinesterase activity of a population of frail hospitalised elderly subjects was shown (Summerbell *et al*, 1993) to be significantly lower than their fit elderly counterparts, although the lower activities of the frail elderly failed to respond to dietary supplementation for a period of eight weeks. These factors are closely related to protein synthesis and, as such, serum cholinesterase appears to reflect them.

It has been shown over the years that serum cholinesterase activity is greater in men than women (Lepage *et al*, 1985; Moses *et al*, 1986) and this has been explained on the basis of hormonal differences. Apart from possible hormonal effects *per se*, the fluctuating hydration status of females through their menstrual cycle could be expected to influence the concentration of plasma proteins. Studies carried out by Mueller *et al*, (1983) and Krisch (1968) have suggested that females have higher paraoxonase activity in comparison to males.

A number of plasma esterases have been shown to be affected by age. Lehman *et al*, (1957) observed plasma cholinesterase activity to be slightly lower in the newborn than in the adult human. After about two months the activity reached adult levels and then continued to increase so that values during childhood were considerably higher than for adults. No significant change in paraoxonase activity was found in individuals aged between three months and seventyeight years (Mueller *et al*, 1983), although activity was reduced in infants less than one year old (Ecobichon and Stevens, 1973).

3.4.3 Acetylcholinesterase

Acetylcholinesterase (EC 3.1.1.7) is found in neurons, at neuromuscular junctions and in some other tissues, such as the red blood cell. It is present on the post-synaptic membrane and on the plasma membrane of the entire cholinergic neuron where its physiological function is to hydrolyse acetylcholine which is released as the result of cholinergic stimulation, so permitting the nerve fibre to accommodate the next impulse. This esterase therefore plays an essential role in the physiology of the nervous system and is present in great excess at cholinergic synapses. Inhibition of acetylcholinesterase will result in the accumulation of acetylcholine in nervous tissue and effector organs and lead to the development of signs and symptoms that mimic the muscarinic, nicotinic and central nervous system actions of acetylcholine.

Hydrolysis of acetylcholine, the endogenous substrate for acetylcholinesterase, is thought to be facilitated by its attachment to two sites recognised in the acetylcholinesterase active centre: the anionic site and the esteratic site. Although the bond formed is highly specific,

acetylation of the enzyme is rapidly followed by breakage of the ester linkage and the elimination of choline. The acetylated enzyme then reacts with water in order to regenerate and release acetic acid. It has been estimated that hydrolysis of acetylcholine by acetylcholinesterase is complete in a few microseconds (Wilson, 1951). The reaction is shown in figure 3.2.

Organophosphorus compounds are potent acetylcholinesterase inhibitors and act by phosphorylating the enzymes' active site; the enzyme is also very sensitive to carbamates including physostigmine, being completely inhibited at concentrations as low as 10^{-6} M. Alkaloids such as prostigmine, cocaine and nicotine are also powerful inhibitors, as are many quaternary ammonium compounds, thiol agents and redox dyes.

Acetylcholinesterase exists as a number of molecular forms, the relative proportions of which vary from tissue to tissue. The various forms appear to be equivalent in catalytic activity but differ in their sensitivity to anticholinesterases, including organophosphates and carbamates (Ogane *et al*, 1992). However, although Volpe and co-workers (1990) found differential inhibition of the various molecular forms in brain by both DFP (O, O diisopropylfluorophosphate) and paraoxon *in vivo*, they found no significant difference *in vitro*. These results were interpreted as differences in the *in vivo* access of the inhibitor to the different molecular forms of acetylcholinesterase.

Sedimentation analysis of rodent diaphragm has shown that the globular monomeric G1 (4s) and tetrameric G4 (10s) forms predominate. The globular 4s and 10s forms are most abundant in diaphragm, although only 26% of the total number is believed to be specifically associated with the

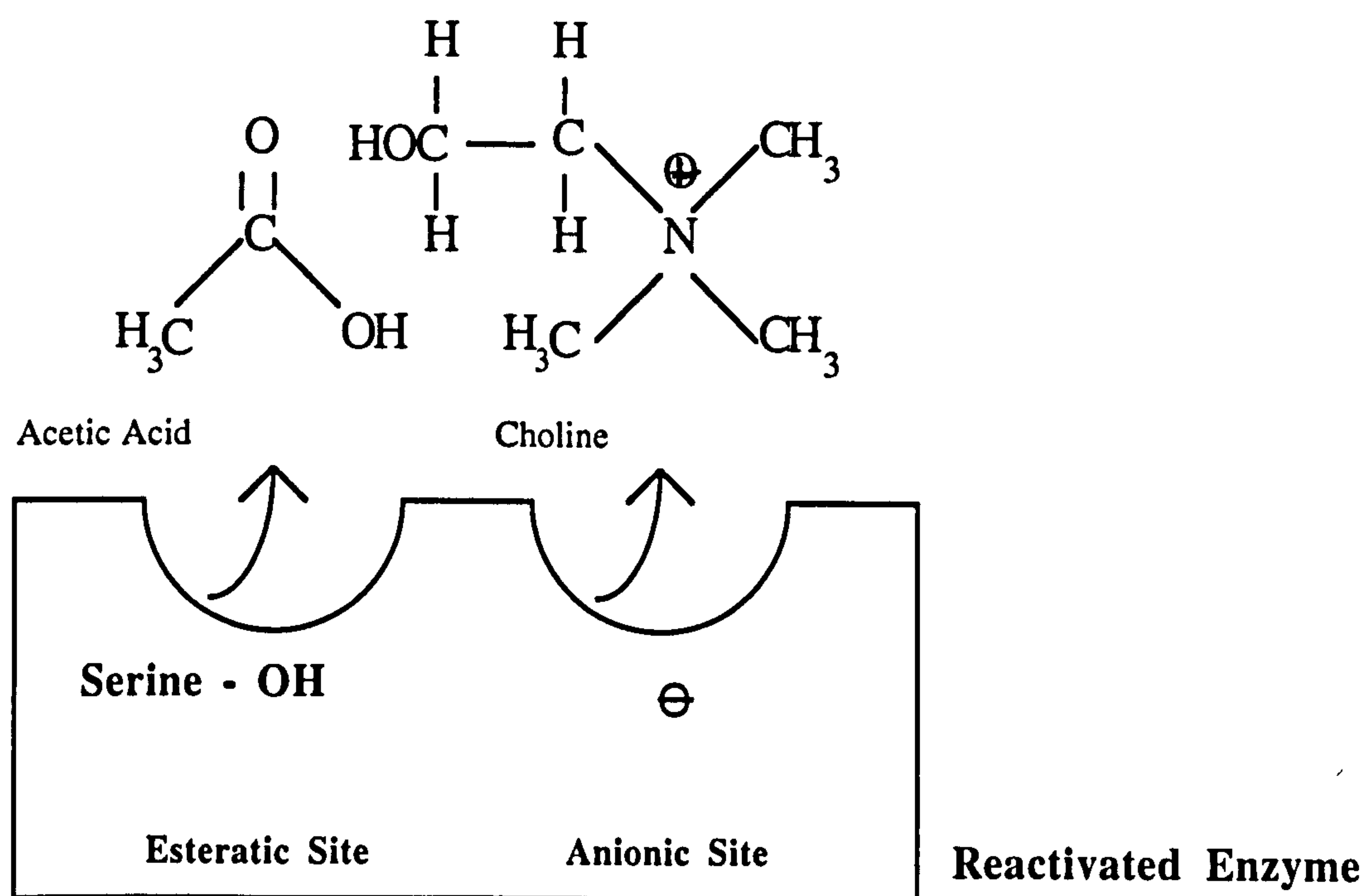
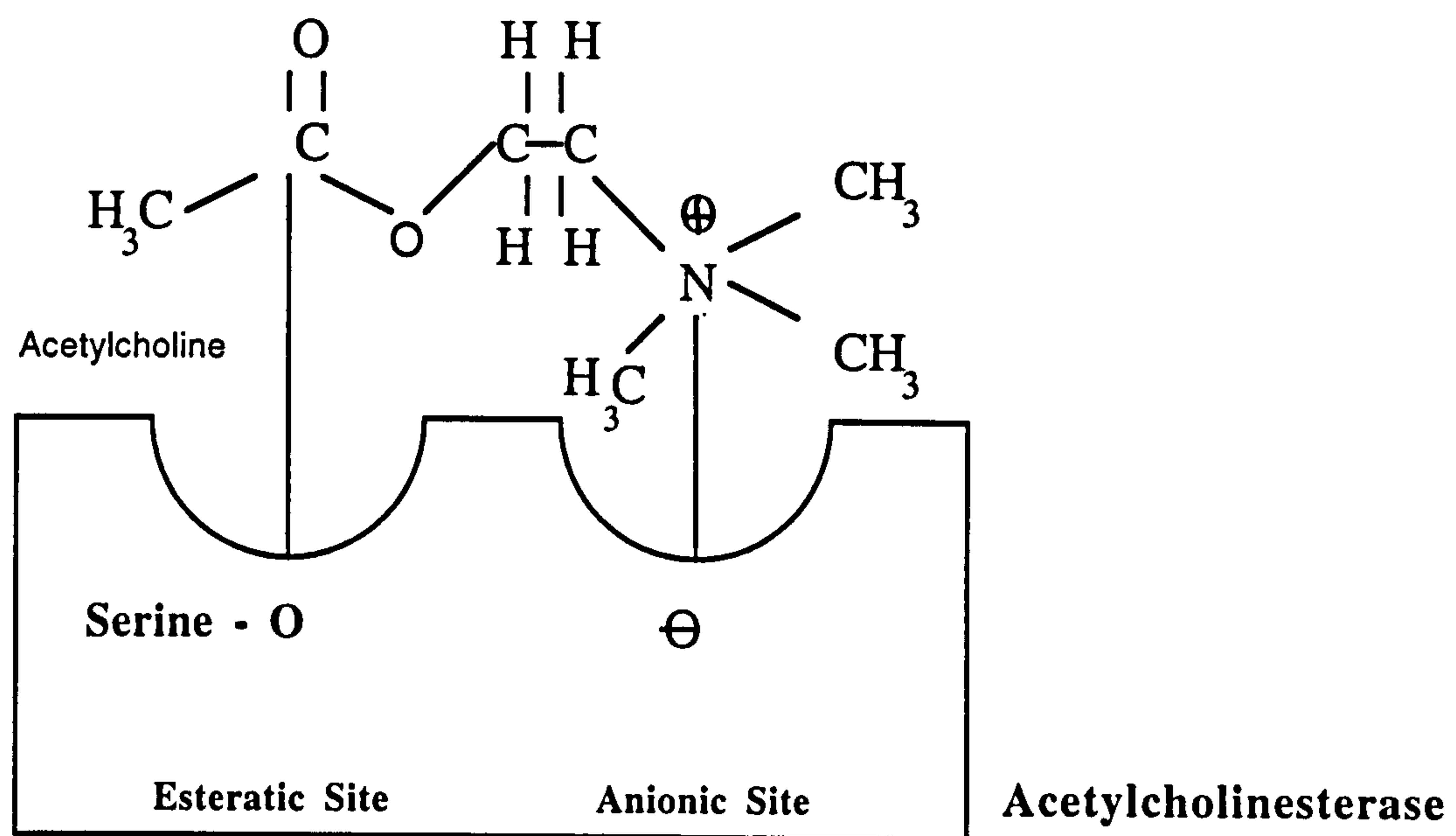


Figure 3.2

Hydrolysis of acetylcholine by acetylcholinesterase resulting in reactivated enzyme.

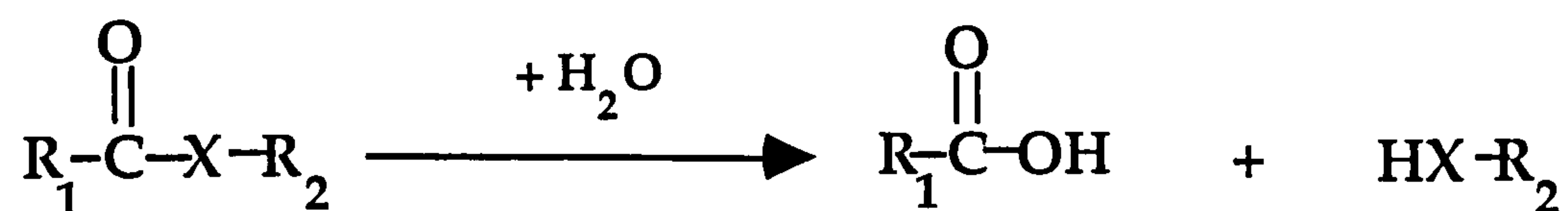
end-plate region (Younkin *et al*, 1982). End-plate specific forms, are predominantly asymmetric A8 (12s) and A12 (16s) structures (approximately 40% are non-extractable) and are associated with the basal lamina of neuro-muscular junctions (Dettbarn, 1984). The A8 and A12 forms have been shown to be mainly external (cell surface) components of diaphragm and it is these sub-populations which are essential in the *in vivo* removal by hydrolysis of acetylcholine from synaptic clefts (Dettbarn, 1984).

Inhibition of red blood cell acetylcholinesterase and plasma cholinesterase by phosphorylation plays an important role in detoxification by removing circulating oxons prior to reaching target tissues. In man, the plasma cholinesterases may influence the available blood concentration of oxon by irreversible binding to the serine active centre of the enzyme. As a metabolic "sink" for organophosphates, plasma cholinesterase will reduce the concentration of agent available for interaction at the neuronal and neuromuscular junctions. Whilst inactivation of red blood cell acetylcholinesterase and plasma cholinesterase does not result in any significant deleterious effects to the individual, monitoring for inhibition of these enzymes is a useful index for organophosphate exposure, with certain limitations.

3.4.4 Carboxylesterases

The carboxylesterases (EC 3.1.1.1) are non-specific serine hydrolases with differing substrate and inhibitor specificities and are present in the membranes and cytosol of most cells. Suitable substrates for measurement of carboxylesterase activity *in vitro* include α -naphthylacetate, phenylacetate and phenylvalerate, although none of these are specific for

the enzyme. The human carboxylesterase, EsD, is distinguishable from other esterases by its' specificity for the hydrolysis of 4-methylumbelliferyl esters. This group of enzymes catalyse the hydrolysis of ester, thioester, and sometimes amide bonds during the course of metabolism of endogenous compounds, environmental contaminants and some drugs. The general mechanism for hydrolysis by carboxylesterases is given in figure 3.3. This generally results in formation of more polar, readily excreted products and therefore constitutes a detoxification process. A list of environmental contaminant esters, and drugs which are detoxified by carboxylesterases are given in table 3.3 and table 3.4, respectively.



X = O, S, or NH

Figure 3.3

Hydrolysis of an ester, a thioester, or an amide to a carboxylic acid and an alcohol, a thiol or an amine, respectively.

Carboxylesterases have been sub-classified according to their isoelectric focussing points (Mentlein and Heymann, 1984) and were shown to have differing substrate specificities with a range of ester compounds. Heymann and Mentlein (1988) then went on to describe four rat carboxylesterases according to their putative natural substrates.

The first carboxylesterase described by Heymann and Mentlein, lysophospholipase, shows five pI bands, but is usually associated with pI band 5.6. It has been identified variously as esterase EA (Arndt *et al*, 1978) or RL2 (Hosokawa *et al*, 1987). Its xenobiotic substrates include phenacetin.

Table 3.3

Some environmental contaminant esters that are detoxified by the carboxylesterases.

Ester Class	Chemical Example	Carboxylesterase
Herbicides	Fluazifop-butyl	Human liver, skin, plasma
Carbamates	Carbofuran	Rat tissues
Pyrethroids	Permethrin, cypermethrin	Rat tissues
Acrylates	Methyl, ethyl, butyl acrylate	Mouse nasal mucosa
Glycol monoalkyl ether acetates	Ethylene, propylene	Mouse nasal mucosa
Trichothecenes	T2 toxin	Rat liver
Nitrosomides	N-nitroso-N-methyl-N-nitroguanidine (MNNG)	Rat liver

Table 3.4

Some drugs that are detoxified by human carboxylesterases.

Drug	Carboxylesterase Source
Aspirin	Purified from human liver, intestinal mucosa
Clofibrate	Purified from human liver, intestinal mucosa
Procaine	Purified from human liver, intestinal mucosa
Cocaine ^a	Purified from human liver
Meperidine	Human liver
Mebeverine	Human plasma
Lorazepam 3-acetate	Human liver

^aHydrolysis of the methyl ester of cocaine.

Long-chain acyl-CoA hydrolase exists as two isoenzyme forms that separate at pIs 6.2 and 6.4. It has been identified (Hosokawa *et al*, 1987) as RL1 and palmitoyl-CoA hydrolase and substrates for this enzyme include aspirin and the phosphorothioate, malathion. Medium-chain monoacyl glycerol lipase is the predominant carboxylesterase in rat liver, its pI value is 6.0. It has been identified as RH1 (Hosokawa *et al*, 1987) and esterase E1 (Arndt *et al*, 1973), and substrates include clofibrate and malathion. The fourth carboxylesterase is diacylglycerol lipase. This enzyme has multiple forms with pI values of 5.0 and 5.2 (Robbi and Beaufay, 1983) and its main xenobiotic substrates are the phorbol esters.

Carboxylesterases predominate in mammals in comparison to the other esterases (Hedrich and von Deimling, 1987) and are also widely distributed in insects (Brooks, 1979). Of the various mammalian organs, the liver and small intestine has the highest carboxylesterase activity, although there is appreciable activity in most other tissues. They are mainly membrane bound, but not exclusively, as demonstrated by McCracken *et al* (1993), who found cytosolic activity for some carboxylesterase substrates. The membrane bound esterases are found lumenally orientated in the endoplasmic reticulum (Mentlein *et al*, 1988; Robbi *et al*, 1990). Human plasma, however, has negligible carboxylesterase activity (Mackness, 1989). Rodents have much higher endogenous levels of carboxylesterases than man, and this difference could partly account for the lower sensitivity of rats to xenobiotics that are more toxic as the ester than as the hydrolysis product (Chambers *et al*, 1991).

The carboxylesterases of non-target tissues play a very important role in the detoxification of some phosphorothioates by covalently binding their oxons to the serine active centre of the enzyme (figure 3.4). In contrast

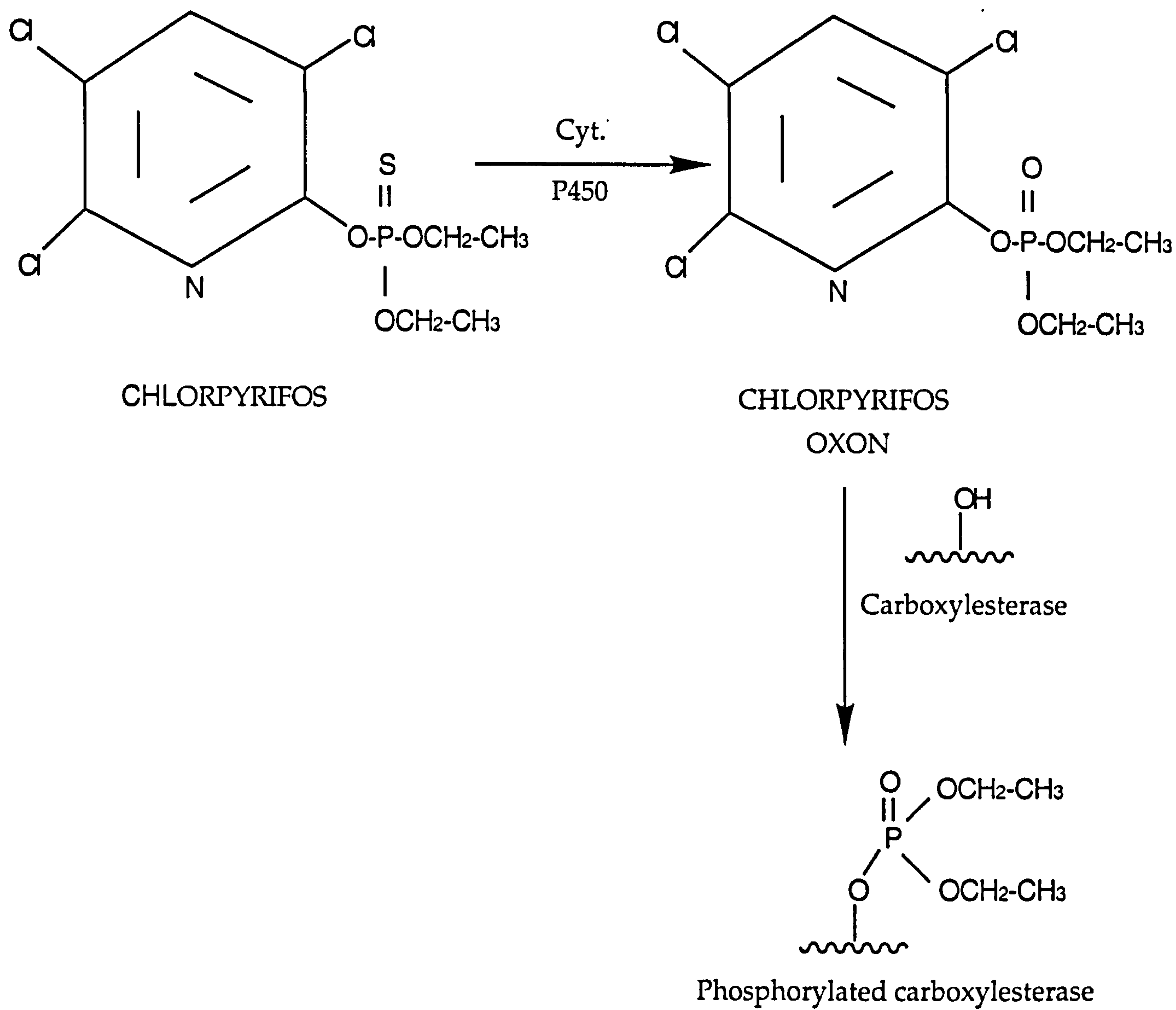


Figure 3.4

The phosphorylation of carboxylesterase by chlorpyrifos oxon.

with the interaction of oxons with A-esterases, dephosphorylation of the B-esterase active centre is very slow and consequently the enzyme is inhibited by the oxon "suicide" substrate. In rodent plasma especially, carboxylesterases function as scavengers which remove circulating oxons before they reach target organs (Gaustad *et al*, 1991). Rodent plasma carboxylesterases will therefore play a very important role in detoxification following organophosphate administration by the sub-cutaneous route, since detoxification will take place concurrently with absorption.

For phosphorothioate pesticides such as malathion the distal carboxylic acid ester is the primary target for hydrolysis by the carboxylesterases, which results in detoxification products containing the corresponding carboxylic acid (figure 3.5). Hydrolytic detoxification by carboxylesterases has been shown to occur primarily in the liver and plasma of mammals, although recent studies have shown that human skin carboxylesterases may also function to detoxify simple phosphorothioates such as malathion (Heymann *et al*, 1993). Once activation of malathion to the oxon has occurred, hydrolysis of the carboxylic acid ester does not occur and detoxification is then by way of binding to the carboxylesterase serine active centre resulting in its' inhibition. Carboxylesterase inhibition has been shown (Chambers and Chambers, 1990) to occur more quickly than inhibition of acetylcholinesterase and therefore serves to protect this physiologically important enzyme at target tissues. Further support for a protective role for carboxylesterases was obtained in a study by Chambers *et al* (1991) who demonstrated that pretreatment of rats with carboxylesterase inhibitors such as bis-*p*-nitrophenyl-phosphate (BNPP) potentiated the toxic effects of phosphorothioates.

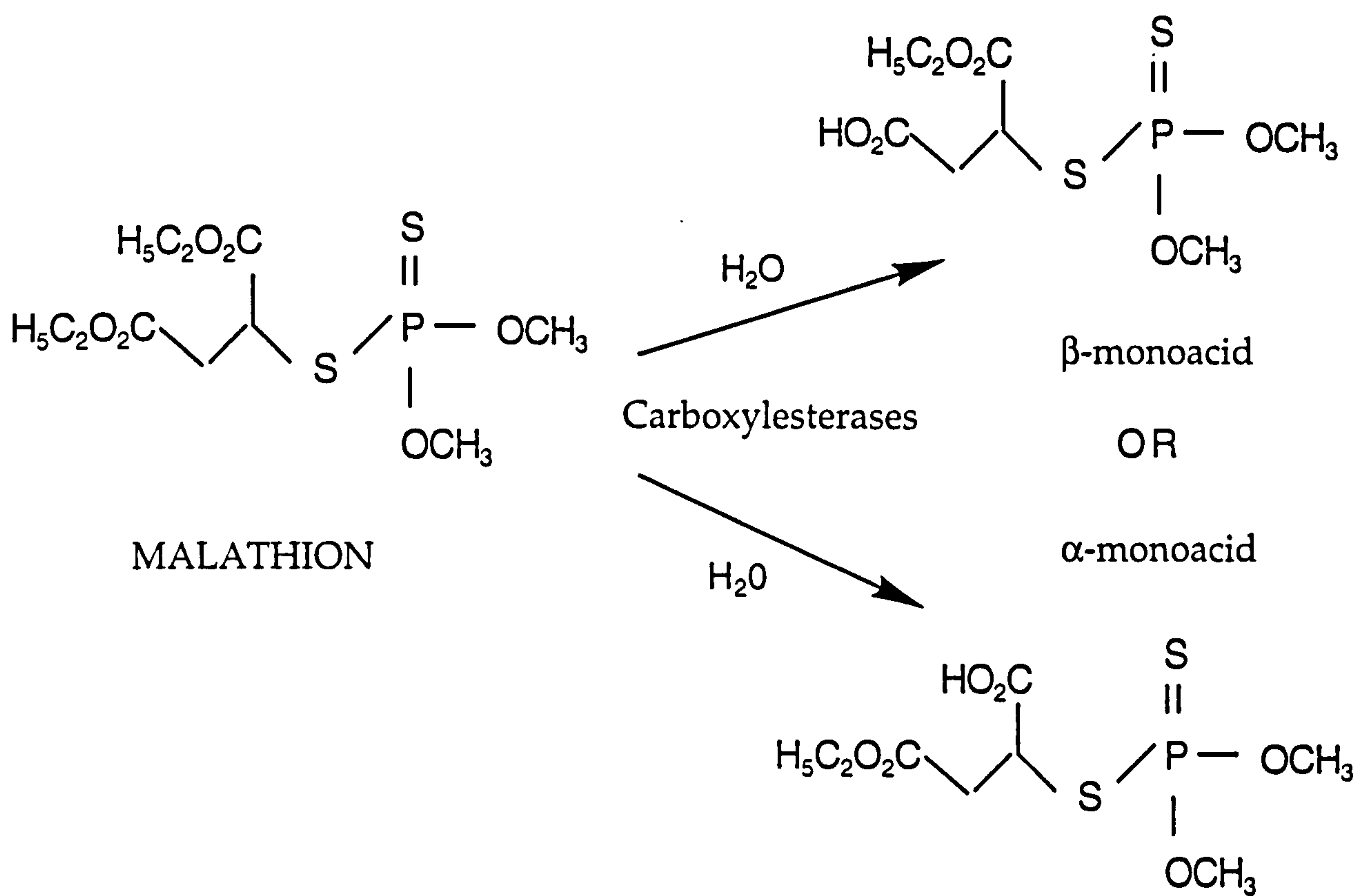


Figure 3.5

The hydrolysis of malathion by carboxylesterases

Hepatic carboxylesterases from rodents have for many years provided a convenient source of a model enzyme, although more recently researchers have been prompted to use the human enzyme directly. A human carboxylesterase (human esterase D, EsD) has been purified from erythrocytes providing a supply of the enzyme so that its' specific role in detoxification may be more fully examined (Okada and Wakabayashi, 1988). Like many of the other human esterases, the physiological function of EsD is not well understood. However, it has been suggested that an endogenous role for the enzyme may involve the reuse of sialic acids since they have been shown to be its' natural substrate (Varki *et al*, 1986).

Human EsD has a molecular weight of 34kDa as determined by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and its' K_m for the substrate 4-methylumbelliferyl was determined to be 10 μ M (Lee *et al*, 1986). Purification of EsD has allowed the preparation of polyclonal and monoclonal antibodies from rabbit and mouse, respectively, that have been useful for immunoquantification studies. More recently (Long *et al*, 1991), the human EsD gene (from human erythrocytes and human liver) has been cloned and sequenced, and comparison of EsD to the genes for human cholinesterase and cholesterol esterase suggest an evolution from a common ancestral gene (Shibata *et al*, 1993). The human liver carboxylesterase has been shown to have significant sequence homology to various mammalian carboxylesterases.

Quantification of EsD in various tissues has been accomplished by using the specific substrate 4-methylumbelliferyl acetate (Lee *et al*, 1986). The results from this study showed that most EsD activity was present in the liver but, perhaps of greater toxicological importance, significant activity was also found in the lung, blood monocytes, skin and nasal mucosa. This

is in agreement with a study conducted by Clark *et al* (1993) who detected carboxylesterase activity in human skin using fluazifop butyl as substrate. Human carboxylesterases therefore appear to be present in all tissues which present themselves as portals of entry for xenobiotics and consequently esterase-mediated detoxification in these tissues may influence the toxicity of potentially toxic esters.

3.4.5 Neuropathy target esterase

Neuropathy target esterase (NTE) is a membrane bound carboxylesterase present in brain and neural tissue, although it can also be detected in most other organs, including adrenal, kidney, liver and spleen (Moretto and Lotti, 1988). The physiological function of this esterase is unknown, but its catalytic activity does not appear to be necessary for the health of nerve axons. NTE has been proposed as the target site at which certain neuropathic organophosphates, such as DFP and mipafox, act to initiate organophosphate-induced delayed neuropathy (OPIDN) (Johnson, 1969; 1982).

Parathion is not believed to cause OPIDN and therefore neuropathy target esterase will be discussed no further.

3.5 Phase 2 enzymes

3.5.1 Glutathione-S-transferases

Glutathione is a tripeptide (cysteine-glycine-glutamate) found in all eukaryotic cells. It has many roles: as a reducing agent and antioxidant, a cysteine reservoir, involvement in the synthesis of some prostaglandins,

and in the conjugation of many xenobiotics. This review will be concerned only with the involvement of glutathione in the conjugation of xenobiotics.

Glutathione-S-transferases (EC 2.5.1.18) catalyse the nucleophilic addition of the tripeptide glutathione to substrates that have electrophilic functional groups. They exist as a family of isoenzymes in the endoplasmic reticulum and cytosol of liver and extrahepatic tissues, each with a unique substrate specificity. The primary function of these enzymes is considered to be the detoxification of both endogenous and xenobiotic alkylating agents such as epoxides, α -, β -unsaturated aldehydes and ketones, and alkyl and aryl halides (Armstrong, 1991). The general reaction equation is given below.



where RX may potentially be any xenobiotic or phase 1 reaction product.

3.5.2 UDP glucuronosyltransferases and sulphotransferases

The most common conjugation pathway for xenobiotics is glucuronidation (Jakoby and Habig, 1980). The enzyme family catalysing glucuronidation is the membrane-bound uridine diphospho-glucuronosyltransferase (EC 2.4.1.17). Conjugation takes place when UDP- α -D-glucuronic acid (UDPGA) and the xenobiotic are at the same enzyme site, but the exact mechanism is not yet understood. Hydroxyl groups are the most commonly conjugated groups, although carboxyl, amino and sulphydryl groups may also be conjugated. The general reaction is given below.



where ROH may potentially be any xenobiotic or phase 1 reaction product.

There exists two UGT families: UGT 1 enzymes are dioxin inducible, whereas the UGT 2 family are not. Marker substrates for UGT 1 enzymes have included *p*-nitrophenol, and bilirubin and testosterone for UGT 2 enzymes (Burchell *et al*, 1994).

Sulphate conjugation occurs in the cytosol, the enzymes catalysing this reaction are the sulphotransferases (EC 2.8.1, 2, 4, 9 or 14). The sulphate conjugating group is donated by 3'-phosphoadenosine 5' phosphosulphate (PAPS) which is generated from ATP and inorganic sulphate. Thirteen forms of rat sulphotransferase have been identified, compared to only five in man (Weinshilboum, 1997). The human forms have been divided into phenolsulphotransferases (P-PST1, P-PST2, M-PST) and hydroxysteroid sulphotransferases (EST and HST). The general reaction is given below.



where ROH may potentially be any xenobiotic or phase 1 reaction product.

There is significant variation in sulphotransferase activity between individuals and there is some evidence that expression is under genetic control. P-PST is the most studied form, with approximately 40 fold variations in activity, and activity has been shown to vary due to several allelic variants in man (Coughtrie, 1996). It has been suggested that low or high sulphotransferase expression may indicate a risk factor with reduced detoxification and increased bioactivation of a xenobiotic.

3.6 The proposed metabolism of parathion

Phosphorothioate pesticides such as parathion are believed to undergo metabolic activation and detoxification as a result of P450-dependent oxidative desulphuration as proposed in figure 3.6 (Nakatsugawa and Dahm, 1967; Kamataki *et al*, 1976). Until very recently, the cytochrome(s) P450 involved in the reaction had not been investigated.

Studies with ^{32}P and ^{35}S labelled parathion and phenobarbital induced rabbit liver microsomes suggested that P450 donates an atom of singlet oxygen to the sulphur moiety of the phosphorothioate to form an unstable phosphooxythiran. This intermediate structure is then believed to spontaneously rearrange to form paraoxon, *p*-nitrophenol and their associated dialkylphosphates. Because of its unstable nature, the phosphooxythiran has never been positively identified.

Production of oxygen analogues (oxons) such as paraoxon represents the metabolic activation of phosphorothioates, since the parent compounds have little or no capacity to inhibit acetylcholinesterase. For example, activation of parathion to paraoxon has been shown to increase its anticholinesterase potential by three orders of magnitude (Forsyth and Chambers, 1989). Since P450-dependent oxidative desulphuration of parathion plays a critical role in controlling the extent of acetylcholinesterase inhibition following exposure, factors that alter the metabolism of such insecticides can potentially alter their acute toxicity.

Rat liver carboxylesterases have been shown to be capable of removing about 11 nmoles paraoxon per gram of tissue by covalent binding which represents a significant means of acetylcholinesterase protection

(Chambers *et al*, 1994). In contrast, detoxification of paraoxon by the hydrolytic A-esterases is catalytic which, in a practical sense, is a non-saturable process *in vivo*, and should therefore not be limited in capacity. The extent of the capacity of the liver to detoxify paraoxon was demonstrated by Sultatos (1986) who showed that relatively little parathion or paraoxon perfused into mouse liver *in situ*, was capable of escaping.

Although the glutathione-S-transferases are known to be effective for parathion detoxification *in vitro*, several researchers have shown that they are not important at biologically relevant concentrations, *in vivo* (Sultatos, 1992; Chambers *et al*, 1994). Glucuronic acid and sulphate have been shown to aid in the elimination of some primary degradation products, such as those produced following ester hydrolysis or oxidation by the cytochromes P450. For example, *p*-nitrophenol, the detoxification metabolite of parathion metabolism, may follow conjugation with sulphate or glucuronic acid prior to excretion in some instances (Sultatos and Minor, 1985).

Chapter 4

Chapter 4 Pharmacogenetic polymorphisms

4.1. Introduction

The early definitions of genetic polymorphism (Ford 1940; Cavalli-Sforza and Bodmer 1971; Vogel and Motulski 1986) were designed to distinguish between common and rare phenotypes, "A polymorphism is a Mendelian or monogenic trait that exists in the population in at least two phenotypes (and at least two genotypes), neither of which is rare - that is, neither of which occurs with a frequency of less than 1-2%" (Vogel and Motulski, 1986). This definition therefore distinguished polymorphisms as common traits from spontaneous mutations, which occur at a much lower frequency. However, it did not specify if the rare phenotype was of heterozygous or homozygous genotype for the variant allele.

Analysis of genomic DNA by restriction fragment analysis on Southern blots or sequencing frequently reveals "DNA polymorphisms", which may be unrelated to a "clinical phenotype" since many of the DNA variations are silent polymorphisms, i.e. they have no consequence at the protein level (Antonarakis 1989). Many of the genetic polymorphisms so defined at the DNA level (genotype) will be of no clinical consequence at the level of protein amount or function, i.e. they will not cause the appearance of different "pharmacological or toxicological" phenotypes in the population.

The majority of xenobiotic-metabolising enzymes show considerable interindividual variation in expression even when they exhibit a unimodal distribution of activity. However, polymorphisms have also been detected for some of these enzymes at both the genotypic and phenotypic level. The major polymorphisms that have received clinical

attention are those relating to the cytochromes P450: CYP2D6 (debrisoquine hydroxylase), CYP2C19 (S-mephenytoin 4' hydroxylase), CYP1A1 (aryl hydrocarbon hydroxylase), CYP1A2 (arylamine oxidations), CYP2E1 (N, N- dimethylnitrosamine N-demethylase). Polymorphisms have also been shown for flavin monooxygenase (trimethylamine N-oxidation), acetylation by N-acetyltransferase (NAT2), S-methylation by thiopurine methyl transferase, conjugation reactions by glutathione S-transferase μ and θ , and ester hydrolysis by plasma cholinesterase and plasma paraoxonase. Examples of these polymorphisms are given in figure 4.1.

It has been suggested (Daly *et al* 1993) that polymorphisms of the xenobiotic-metabolising enzymes have occurred because these enzymes lack a normal metabolic role and are only of importance when the individual is challenged either by environmental xenobiotics or by drug administration. The author bases this hypothesis on the discovery that wide interindividual variations have not been detected in enzymes with essential physiological roles.

The pharmacokinetic and pharmacodynamic consequences of the activity of a polymorphic enzyme depend upon whether it mediates metabolism of parent drug or primary metabolite (or both), and whether parent drug or primary metabolite (or both) are active. Furthermore the consequences will reflect the enzyme's overall contribution to clearance from the affected pathway, the potency of the active species, and the influence of competing pathways of elimination.

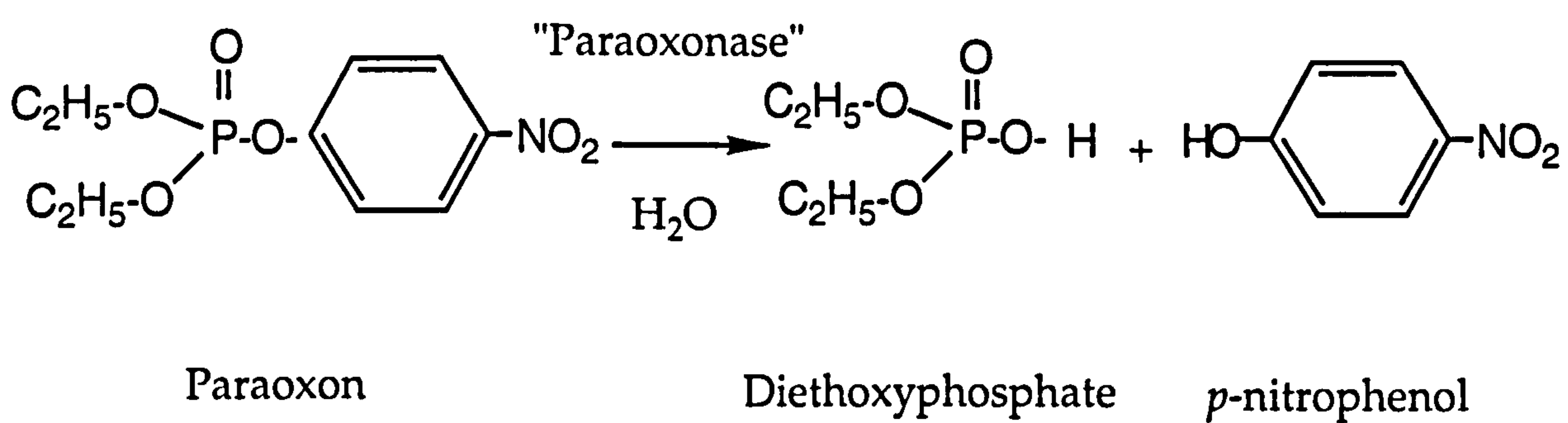
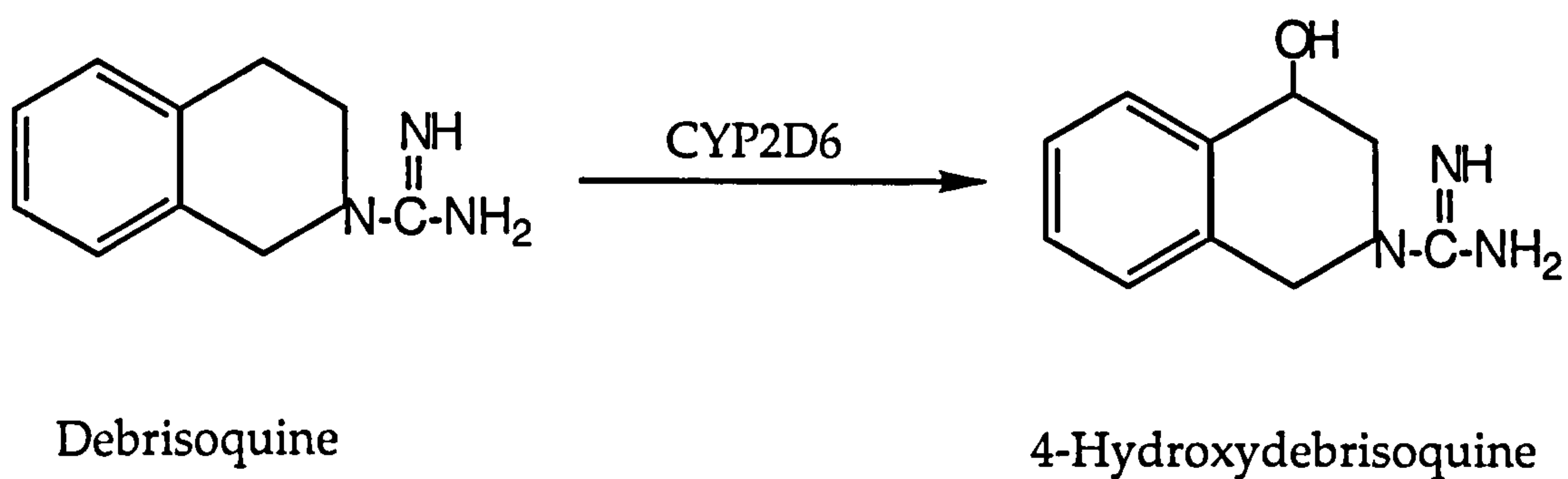
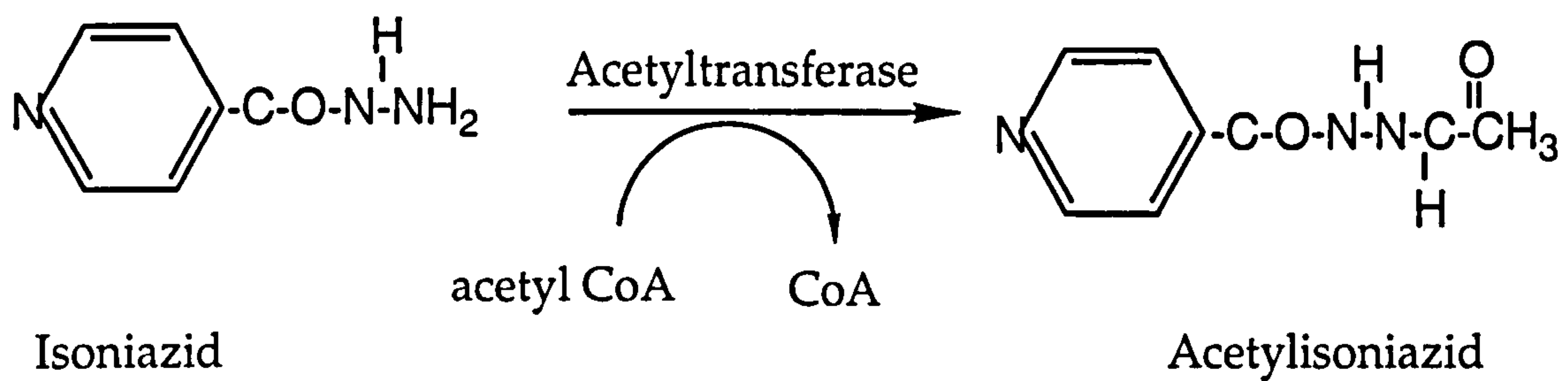


Figure 4.1

Enzymes that demonstrate polymorphic expression in the human population

The clinical significance of the interindividual variability in the activity of an enzyme relates to possible acute drug toxicity and drug-drug interactions, such as seen with the immunosuppressive drug cyclosporine A which is metabolised by CYP3A. The daily dose necessary to achieve a target blood level for this drug has been demonstrated to vary at least tenfold among different patients, necessitating monitoring of plasma levels during treatment (Watkins *et al* 1990). It has also been reported that some transplant patients who had received inhibitors of CYP3A, such as ketoconazole, had pronounced increases in their cyclosporine A blood level, resulting in potentially serious central nervous system and kidney toxicity. In addition, chronic effects have been demonstrated. Over one's lifetime, differences in the metabolism of drugs, occupational chemicals and pesticides, and other environmental pollutants can lead to interindividual differences in the buildup of DNA damage (e.g. mutations, chromosomal breaks) and nongenotoxic signals (e.g. signal transduction pathways without DNA damage) leading to toxicity, and tumor initiation and progression.

The extent and implications of interindividual variations in the cytochromes P450 2D6, 2C19, 2C8/9, 1A2, 1A1, 2A6, 2E1, 3A4/5 and the FMOs will be discussed in this chapter since they are of potential importance in the activation of parathion.

4.2 Xenobiotic-mediated polymorphisms

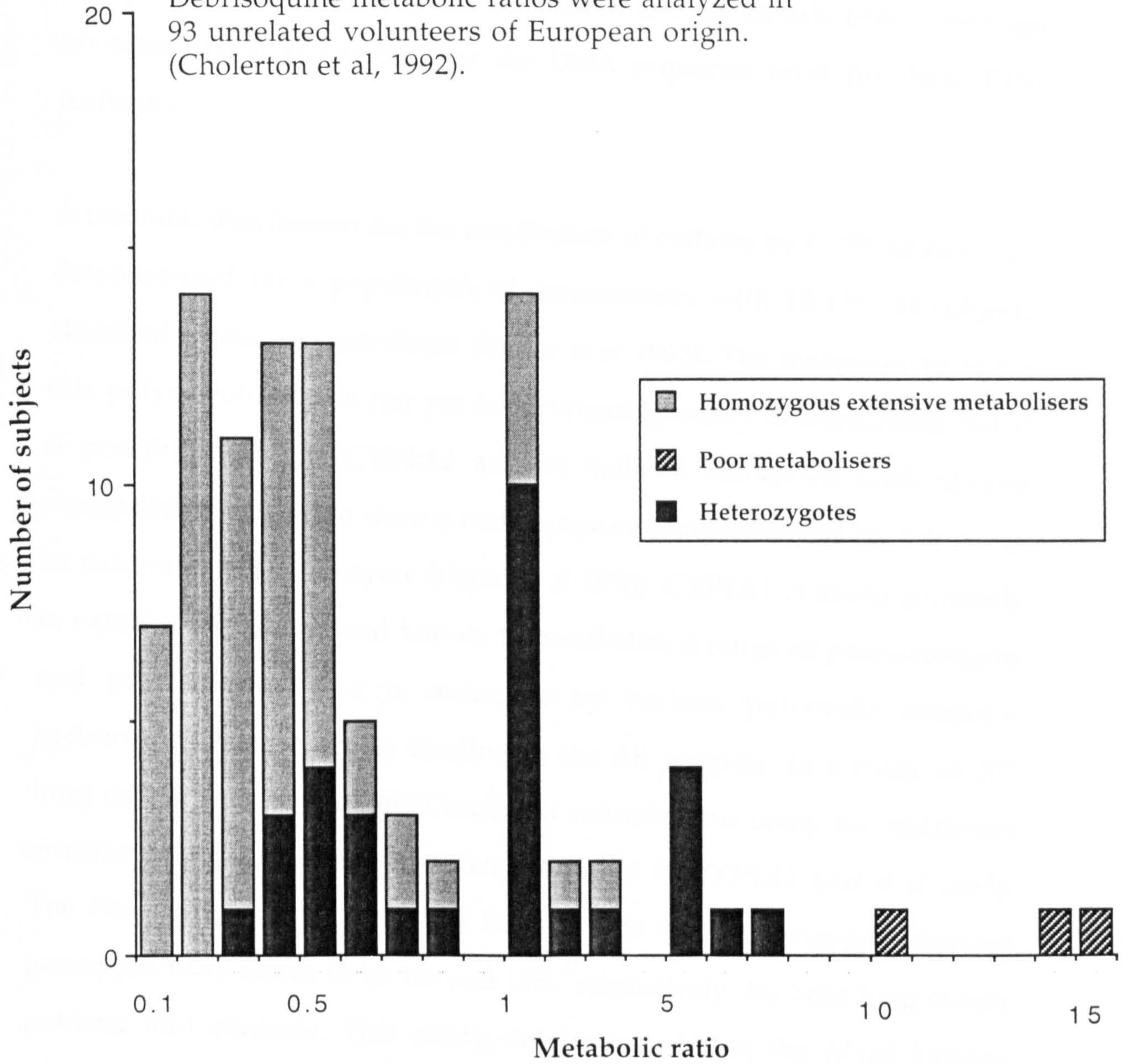
By far the best studied polymorphism is that of debrisoquine hydroxylation by CYP2D6. Debrisoquine and Sparteine are commonly used to probe the activity of CYP2D6 *in vivo* and *in vitro*. For both drugs two phenotypes can be discerned, extensive and poor metabolisers (EM and

PM, respectively) with 5-10% of Caucasian PMs not expressing this enzyme (Idle, 1991). Previous pharmacological studies of the debrisoquine polymorphism have used metabolic phenotyping for the identification of poor metabolisers. These studies required administration of the drug followed by quantitation of the urinary metabolites produced. In contrast, genotyping methods require a small blood sample which can be taken at any time. A clear relationship has been demonstrated between genotype and phenotype for debrisoquine hydroxylase in ninety-three Caucasian volunteers and is shown in figure 4.2 (Cholerton *et al* 1992). The potential clinical significance of the CYP2D6 polymorphism relates mainly to drugs acting on the cardiovascular and central nervous systems e.g. flecainide, mexiletine, propranolol, thioridazine, amitriptyline. Some of the drugs metabolised by CYP2D6 have a narrow therapeutic index (e.g. mexiletine) and there is therefore considerable potential for an adverse clinical outcome in certain cases. There is some evidence that the poor metaboliser phenotype is associated with more adverse effects, particularly sedation from neuroleptics, and many of these drugs are potent inhibitors of CYP2D6.

The 4'-hydroxylation of S-mephenytoin by the cytochrome P450 CYP2C subfamily exhibits a genetic polymorphism in humans, with individuals being characterised as either extensive or poor metabolisers (Inaba 1989). The PM phenotype has a higher frequency in Oriental populations (18-23%) compared with Caucasians (3-5%) (Bertilsson *et al* 1992). After many years of effort CYP2C19 was finally identified as the principal S-mephenytoin hydroxylase in humans (Goldstein *et al* 1994) and the molecular basis of the polymorphism has now been clarified (de Morais *et al* 1994). Metabolism of a number of therapeutically important drugs has

Figure 4.2

The relationship between debrisoquine metabolic ratio and genotype. Debrisoquine metabolic ratios were analyzed in 93 unrelated volunteers of European origin. (Cholerton et al, 1992).



been shown to co-segregate with mephenytoin metabolism eg hexobarbital, omeprazole, propranolol, imipramine and proguanil. Another member of the CYP2C subfamily, CYP2C9, has been implicated in a rare polymorphism for the metabolism of tolbutamide (Page *et al* 1991) and possibly also of phenytoin (Goldstein and de Morais 1994), although no information is available at the DNA sequence level for these P450 isoforms.

A trimodal distribution for the metabolism of caffeine by CYP1A2 has been demonstrated for a population of non-smokers with 12-13% of subjects classified as slow metabolisers (Butler *et al* 1992). The molecular basis for this polymorphism has not yet been unambiguously demonstrated but it is possible that low CYP1A2 activity will be associated with altered susceptibility to disease since a wide range of procarcinogens are known to be substrates for the enzyme (Eaton *et al* 1995). CYP1A1 is found primarily in extrahepatic tissues and known to metabolise a range of procarcinogens and promutagens, and is inducible by various polycyclic aromatic hydrocarbons which act by binding to the Ah receptor. In a study of 207 lung cancer patients and 283 Caucasian controls, and using the restriction enzyme *MspI*, a polymorphism was detected in *CYP1A1* (Xu *et al* 1996). The frequencies of the the *MspI* homozygote and heterozygote variant genotypes were found to be 1% and 17%, respectively, for both lung cancer patients and controls. This study demonstrated that the *MspI* variant *CYP1A1* genotype is significantly associated with an increased risk of lung cancer after controlling for confounding factors and, moreover, an elevated risk was noted in heterozygotes.

CYP2A6 carries out the 7-hydroxylation of coumarin and an inactive variant (*CYP2A6v*) appears to be associated with a reduced recovery of

metabolites (Daly *et al* 1994). A recent study by Hu *et al* (1997) of almost 200 individuals described several rare mutant alleles of *CYP2E1*, one of which caused reduced protein expression and a 60% reduction in catalytic activity towards the probe substrate, chlorzoxazone. This polymorphism is of major toxicological interest since *CYP2E1* is known to metabolise several procarcinogens, drugs and solvents to reactive metabolites.

A polymorphism in flavin monooxygenase activity was demonstrated in 169 white British subjects using the dietary component triethylamine (Cholerton and Smith, 1991). One of these subjects (0.6%) was shown to excrete significantly increased concentrations of free triethylamine in their urine and, since this amine has a strong fish-like odour, said to suffer from "fish-odour syndrome". Over 5000 people in Great Britain are believed to have this syndrome (Waring and Emery, 1995). The N-oxidation of triethylamine, to the non-odorous metabolite triethylamine N-oxide, is mediated by flavin monooxygenase and the variant allele appears to give rise to a form of flavin monooxygenase with reduced activity with respect to this pathway (Higgins *et al* 1972). Flavin monooxygenase-mediated metabolism of a wide group of agricultural chemicals, such as aldicarb, methiochlor and diethyldithiocarbamate, has also been observed (Hodgson and Levi, 1992). Although oxidative desulphuration of phosphorothioates is not directly catalysed by the flavin monooxygenases, the thiono group of fonofos (S-phenyl ethyl ethylphosphonodithioate), and other phosphorothioates containing a phosphorus-carbon bond, appears to be metabolised by these enzymes leading to production of the active oxon (Smyser and Hodgson, 1985). The extent of interindividual variability in flavin monooxygenase-mediated capacity to activate phosphorothioate pesticides, has not been determined.

4.2.1 The cytochrome P450 3A subfamily

Cytochrome P450 3A is the most abundant subfamily in human liver and accounts for about 30% of the total P450 pool as determined immunochemically (Shimada *et al* 1994). CYP3A3, originally purified by Watkins *et al* (1985) and termed 'HLp', and CYP3A4, purified by Guengerich *et al* (1986) and termed P450_{NF}, were originally presented as distinct isoforms. CYP3A3 was then shown to be 98% homologous with CYP3A4 (Nelson *et al* 1993) but no significant differences in catalytic properties were identified (Aoyama *et al* 1990). CYP3A3 has been recently discontinued and it is therefore assumed that CYP3A3 and CYP3A4 are the same isoform, i.e. CYP3A4 (Nelson *et al*, 1996). Another CYP3A enzyme, CYP3A5, appears to be detectable in approximately 20% of adult human livers (Wrighton *et al* 1989) and has been found to be 90% homologous with CYP3A4 (Aoyama, 1989).

Several reports have suggested that CYP3A4 and CYP3A5 have a similar, but not identical, substrate specificity. It therefore seems reasonable to suggest that those livers expressing the polymorphic CYP3A5 along with CYP3A4 would influence the wide range in activities observed for those xenobiotics, eg nifedipine (Aoyama *et al* 1989) and midazolam (Gorski *et al* 1994), which are metabolised by both isoforms.

CYP3A isoforms are also abundant in the intestinal mucosa and have a six-fold variation in catalytic activity (midazolam hydroxylation, Lown *et al* 1994). There was an excellent correlation ($p=0.0001$) between intestinal CYP3A4 protein level and catalytic activity for the patients studied ($n=20$), although neither parameter significantly correlated with hepatic CYP3A4

activity. CYP3A5 protein was also found in the small intestine of 70% of these patients.

A recent study (Haehner *et al* 1996) of a panel of human kidney microsomes (n=27) showed a bimodal distribution for midazolam hydroxylase (CYP3A) activity which was also suggested by the distribution of CYP3A4 and CYP3A5 proteins. All kidneys expressed CYP3A5 protein but, in contrast to hepatic tissue, CYP3A4 was detected in only 28% of the samples. The authors hypothesised that the bimodal distribution of CYP3A5 may represent induction of this isoform in a select population and/ or a genetically determined organ-specific pattern of expression.

A fourth identified member of the human CYP3A subfamily is termed CYP3A7. CYP3A7 protein was first isolated from human foetal liver where it is the major P450 present (Kitada *et al* 1988), although the mRNA for this isoform has more recently been detected in approximately 50% of adult human livers (Schuetz *et al* 1994). It is, however, uncertain whether CYP3A7 protein is expressed in adult liver. In addition, it has been reported that CYP3A7, but not CYP3A4 or CYP3A5, protein is expressed in the human endometrium and placenta (Schuetz *et al* 1993). The substrate specificity for CYP3A7, unlike CYP3A4 and CYP3A5, remains poorly characterised but it is possible that this isoform plays an important role in the metabolism of specific steroid precursors and/or xenobiotics in order to protect the foetus from toxic insult.

The list of drugs metabolised chiefly by CYP3A is expanding rapidly and includes several drug categories, including immunosuppressants, calcium channel blockers, cancer therapeutic agents, antihistaminics, sedatives, and synthetic estrogens. Indeed, it has been estimated that up to one-third

of the orally administered pharmaceuticals currently in use, and up to one-third of the new chemical entities currently under development, are capable of being metabolised by CYP3A (Inaba *et al* 1995). Table 4.1 gives a list of drugs known to be metabolised by CYP3A.

Wide interindividual variations are seen in the metabolism of all CYP3A substrates and this may account, at least in part, for differences amongst patients in response to these, and other, medications metabolised by the CYP3A subfamily. In addition to the variation in expression in CYP3A4 and the polymorphic expression of CYP3A5, it appears that some drug interactions result from concurrent administration of other drugs, or dietary components (eg the natural flavonoids naringenin and quercetin) which are also metabolised by CYP3A. Furthermore CYP3A4, but not 3A5 (Wrighton *et al* 1989), is highly inducible by many medications in common use, such as dexamethasone, phenobarbital and rifampicin, and this will further influence the level of variability observed in the CYP3A-mediated metabolism of xenobiotics.

A study by Hoyo-Vadillo *et al* (1989) of the *in vivo* metabolism of nifedipine demonstrated bimodal distributions for the recovery of metabolites and provided some evidence for a polymorphism in CYP3A at the phenotypic level. Other *in vivo* studies with nifedipine (Renwick *et al* 1988; Schellens *et al* 1988) have failed to confirm this bimodality, although again a considerable variability in recovery of urinary M-II metabolite was observed. The isolation and sequencing of cDNA clones for CYP3A4 have now been carried out and the gene, CYP3A4, has been localised to human chromosome 7q (Gonzalez *et al* 1988). Chromosome 7q also contains the defective cystic fibrosis (CTFR) gene, although it appears to be only weakly linked to CYP3A4 (Brooks *et al* 1988). A study by Daly *et al* (1992) showed

Table 4.1
 Use or occurrence of chemicals metabolised by human hepatic cytochrome P450 3A4.

XENOBIOTIC	USE/OCCURRENCE
Acetaminophen	Analgesic
Aflatoxin B ₁	Food hepatocarcinogen
Alfentanil	Opioid anaesthetic
Amiodarone	Antiarrhythmic
Androstenedione	Steroid
Benzo α pyrene	Combustion product
Caffeine	Dietary
Cocaine	Narcotic
Cortisol	Hormone
Cyclophosphamide	Anticancer
Cyclosporine	Immunosuppressant
Dextromethorphan	Antitussive
17 α -Ethinylestradiol	Oral contraceptive
Estradiol	Steroid hormone
Ifosfamide	Anticancer
Imipramine	Antidepressant
Lidocaine	Anaesthetic; antiarrhythmic
Lovastatin	Cholesterol-lowering
Nifedipine	Calcium channel blocker
Progesterone	Steroid
Terfenadine	Antihistamine
Testosterone	Steroid
Zonisamide	Anticonvulsant

Reference
 List compiled by AP Li *et al* (1995). Toxicology 104: 1-8.

that a group of cystic fibrosis patients and their first-degree relatives exhibited significantly slower metabolism of nifedipine compared with a control group and that there was no evidence for bimodality in the controls.

Interethnic differences in capacity to metabolise nifedipine have also been observed. A study by Ahsan *et al* (1991) compared the pharmacokinetics of a single dose of nifedipine (20mg) in five South Asian volunteers with data for twentyseven Caucasian volunteers. A threefold higher area under the plasma drug concentration-time curve (AUC) was shown in this study for the Southern Asians when compared with Caucasians, although this difference was not verified for triazolam, another CYP3A substrate (Kinirons *et al* 1996). In the Asian study it was noted that consumption of a spicy curry diet for three days by the Caucasians did not significantly affect the pharmacokinetics of a single dose of nifedipine. These data suggest that the large inter-ethnic differences in nifedipine clearance are not generalisable to all CYP3A substrates but the treatment of patients of South Asian origin with nifedipine should be initiated with lower doses than would be given to Caucasians.

Chapter 5

Chapter 5 Factors influencing phosphorothioate toxicity

5.1. Introduction

The availability of phosphorothioates for metabolism will be influenced by the physicochemical properties of the chemical such as its lipophilicity, stereoisometric form, and protein and tissue binding affinity. Several physiological and environmental factors will also influence metabolism. Physiological factors include polymorphisms of the xenobiotic metabolising enzymes (discussed in chapter 4), interethnic variations, gender, age and hormonal status. Altered enzyme activity may also occur as a result of external factors such as exposure to inducers/ inhibitors of the drug metabolising enzymes. The route of absorption will also be an important consideration since this will influence the rate of delivery of the compound. In addition, first pass extrahepatic metabolism may be of significance in some cases.

All of these factors will be discussed in the following chapter. It should be noted that the influence of these modifying factors on phosphorothioate metabolism has been poorly investigated. The majority of existing data is derived from studies of drugs.

The above modifiers contribute to variations in the kinetic parameters of enzymes involved in the metabolism (activation and detoxification) and thence toxicity of phosphorothioates. The kinetic parameters and metabolic pathways of the enzymes involved in phosphorothioate toxicity were discussed in chapters 2 and 3, respectively.

5.2 Physicochemical properties

There is no evidence of prolonged storage of phosphorothioates in the body, since they are very quickly degraded by the metabolic reactions previously described. In this context, an *in vitro* study by Sultatos *et al* (1985) showed that incubation of various concentrations of paraoxon with heparinised mouse blood resulted in its detoxification to *p*-nitrophenol by apparent first-order kinetics with an average elimination half-life for paraoxon of 8.6 minutes. The rapid elimination half-life for paraoxon was corroborated by experiments in which mouse livers were perfused *in situ* with the parent phosphorothioate, parathion, in a perfusate containing 4% bovine serum albumin (Sultatos and Minor, 1986). In these studies the half-life associated with the approach to steady state of parathion was 6.2 ± 0.4 minutes, under which conditions the fraction of unbound phosphorothioate was 4%.

Phosphorothioates are less lipophilic than organochlorine pesticides as denoted by their relatively lower log octanol/ water coefficients. For example, chlorpyrifos and parathion have coefficients of 4.7 and 3.0, respectively (The Agrochemicals Handbook, 1991), while the organochlorine pesticide DDT (1,1,1-trichloro-2,2-bis[4-chlorophenyl] ethane) has a value of 6.2 (Moody, 1993). Organochlorine pesticides are known to accumulate in adipose tissue and the environment and are poorly metabolised. However, although phosphorothioates are extensively metabolised once in the general circulation, limited storage in fat of the more lipophilic compounds (eg chlorpyrifos) may somewhat delay excretion in some cases. The elimination half-life for chlorpyrifos (dermal application, human) and diazinon (i.p., rat) has been shown (Fenske and Elkner, 1990; Wu *et al*, 1996) to be about 27h and 4.5h,

respectively, with no unchanged phosphorothioate or oxon detected in the urine.

5.3 Physiological factors

There exists a considerable body of information regarding the cytochromes P450 in rats, although the human forms have been less extensively studied. It should be noted that the impact of the modifying factors to be discussed in this chapter are different for animals and humans. It is therefore important to know how these factors influence metabolism of the compound of interest in order to allow more effective extrapolation from animal and human data obtained *in vitro*, and thence to the human population. In addition, identification of species differences for a particular xenobiotic will help ascertain a suitable animal model for man.

Numerous studies (Waxman, 1988; Lee and Werlin, 1995) have shown that the activities of some of the cytochrome P450 isoforms in animals are subject to the influence of modifiers such as strain, species, gender, age and diurnal rhythm. Of these strain, species and gender appear to be the most effective. For humans, genetic predisposition is probably most important (discussed in chapter 4), although the influence of inducing/ inhibiting xenobiotics and nutritional status (for esterases) will considerably contribute to interindividual variations and may mask a genetic polymorphism. Age and gender seem to be of minor impact for man.

5.3.1 Interethnic differences

Interethnic differences in drug response have been recognised for many years, and the international pharmaceutical industry, as well as many

regulatory agencies, pay increasing attention to these differences in pharmacologic and toxic response characteristics (Hahn, 1992). Particular reference to ethnic differences in the cytochromes P450 and esterases involved in the metabolism of phosphorothioates will be made in this chapter since the use of these pesticides is far greater in developing countries with a concurrent increase in potential for exposure compared to the UK.

The cytochromes P450 2D6, 2C9 and 3A4 have provided most information of interethnic differences in enzyme activity. The incidence of PMs of debrisoquine, a CYP2D6 substrate, was found to be very low (1.01%) in four different Chinese nationalities compared to Caucasians (Bertilsson *et al*, 1992). A similarly low incidence of PM has been demonstrated in studies of Japanese (Nakamura *et al*, 1985) and Korean (Sohn *et al*, 1991) populations. Bertilsson and associates (1992) also demonstrated that the distribution of the urinary debrisoquine/ 4-hydroxydebrisoquine metabolic ratio (MR) was shifted to the right for his population of Chinese EMs compared to Swedish EMs. These results suggest that the mean rate of debrisoquine hydroxylation is lower in Chinese EMs compared with Caucasian EMs. This corroborates the results of a study by Rudorfer *et al* (1984) who gave a single dose of desipramine, a CYP2D6 substrate, to sixteen Caucasian and fourteen Chinese healthy subjects. The mean total plasma clearance of the antidepressant was found to be higher in Caucasians than in Chinese. These data, and those of others (eg Lin *et al*, 1991), indicate that Chinese metabolise antidepressants more slowly than do Caucasians, which is consistent with the right shift of the debrisoquine MR in Chinese discussed above.

Several studies (Alvan *et al*, 1990; Wilkinson *et al*, 1992) have shown that the incidence of PMs of S-mephenytoin in Caucasians is about 3%. In contrast, the incidence of PMs of S-mephenytoin in Japanese (Horai *et al*, 1989), Chinese (Horai *et al*, 1989), Koreans (Sohn *et al*, 1992) and Black subjects (Pollock *et al*, 1991) is much higher: 23%, 17.4%, 12.6% and 18.5%, respectively.

Interethnic differences in the disposition of substrates of CYP3A have been studied in populations of limited size. A study by Ahsan *et al* (1991) compared the pharmacokinetics of a single dose of nifedipine (20mg) in five South Asian volunteers with data for twentyseven Caucasian volunteers. A threefold higher area under the plasma drug concentration-time curve (AUC) was shown in this study for the Southern Asians when compared with Caucasians, although this difference was not verified for triazolam, another CYP3A substrate (Kinirons *et al*, 1996). Similar results using nitrendipine were shown in Japanese compared with Caucasians (Fujimura *et al*, 1989). In the Asian study it was noted that consumption of a spicy curry diet for three days by the Caucasians did not significantly affect the pharmacokinetics of a single dose of nifedipine.

These data suggest that the large interethnic differences in nifedipine and nitrendipine clearance are not generalizable to all CYP3A substrates, however the treatment of patients of South Asian origin with dihydropyridine calcium blockers should be initiated with lower doses than would be given to Caucasians. These studies taken together indicate that Asians have lower CYP3A activity and/or level than Caucasians. Whether this possible interethnic difference is genetic or environmental in origin is unknown. CYP3A is inducible and can also be inhibited by low

levels of certain dietary components, and therefore differences in, for example, diet could contribute to interethnic variations.

The worldwide distribution of serum paraoxonase activity has been investigated (Geldmacher-von-Mallinckrodt and Diepgen, 1988), although it should be emphasised that these data were based on measurements of only paraoxon hydrolysis, and only saturating substrate concentrations were used. These data suggest that paraoxonase activity divides European populations into about half with slow and half with rapid activity, but everywhere else the slow variant appears to be the more rare. However, a study by Williams *et al* (1993) showed that serum paraoxonase levels of male Caucasians were higher than male Ghanaians. Similarly, Williams *et al* (1986) found arylesterase (phenylacetate esterase) activity to be lower in Ghanaians compared to a group of Caucasian subjects.

The silent phenotype for cholinesterase, which gives rise to extremely low levels of activity, occurs in about 1:100 000 Caucasians but a relatively higher incidence of this enzyme was found in Alaska and some regions of India (Whittaker, 1986). Studies of aspirin esterase and cholinesterase in Nigerians at home and in the UK showed increased albumin levels when in the UK which were associated with increased esterase levels (Isah *et al*, 1988). This indicates that diet, rather than interethnic differences, influence plasma albumin levels and thence aspirin esterase and cholinesterase activity.

Although very difficult to substantiate, it is possible that the interethnic differences which exist in esterase activity are mainly due to environmental factors such as nutrition (Kalow, 1982), especially since

paraoxonase and cholinesterase have been shown to be closely linked with high and low density lipoprotein, respectively.

5.3.2 Other physiological modifying factors

In general terms, children and young animals are more susceptible than adults to poisons in food. The most common reason is simply because the young eat more in proportion to their weight. Thus, when given the same contaminated food, young animals receive a higher dose of the contaminant.

However, other factors are involved. It is well known that the cytochromes P450 are present at comparatively low levels in neonatal animals, but activity develops to approximate the adult level early in maturation (Fouts and Deveroux, 1972). An extensive study by Cazeneuve *et al* corroborated the evidence of others (Kitada *et al*, 1988) that CYP3A activity is present in foetal liver, but that neither CYP1A1 or CYP1A2 is present at this time. In the Cazeneuve study foetal liver was shown to have the capacity to C-8 hydroxylate caffeine, mainly a CYP1A2 substrate in adult liver microsomes. This suggested that the metabolism of this drug depends on CYP3A at the foetal stage of development.

It is recognised (Waxman, 1988) that the cytochrome P450 families CYP2 and CYP3 are sex-specific, rat P450s 2A2 and 3A2 are male expressed enzymes and 2A1 is a female expressed form. This has been shown to have an important influence on the clearance of drugs metabolised by these P450 families, but the effects are complex and not easily summarised. A recent study by Mahnke *et al* (1997) demonstrated that rat CYP3A genes are variably expressed depending on age and sex. Analysis of mRNA

derived from four CYP3A genes, CYP3A2, CYP3A9, CYP3A18 and CYP3A23, showed that CYP3A2 and CYP3A9 gene expression was age- and sex- dependent, whereas CYP3A18 and CYP3A23 mRNA were observed before and after puberty at fairly constant levels. CYP3A18 and CYP3A23 mRNA levels were about 20% higher in males than females, whereas CYP3A9 mRNA was detected only in adult rats, with a nearly twofold higher expression in females.

Many pharmacokinetic investigations of the elderly reveal decreased clearance of lipophilic drugs metabolised by the cytochromes P450. For example, the clearance of quinidine, midazolam, triazolam, erythromycin and lidocaine, which are metabolised mainly by CYP3A, have been shown to decline with age. However, studies *in vitro* (Hunt *et al*, 1992a) and *in vivo* (Hunt *et al*, 1992b) have established that the activity of CYP3A is stable throughout normal ageing (27-83 years) although it is about 24% higher in females than males. These studies suggested that age-related changes in clearance of CYP3A substrates is secondary to changes in liver blood flow, liver size, drug binding and distribution with ageing.

Hooper and Qing (1990) investigated the effects of both age and gender on the stereoselective elimination of racemic mephobarbital in adult humans. The apparent total body clearance of *R*-mephobarbital, a CYP2C19 substrate, was found to be much greater, and the elimination half-life much shorter, in young men (18-25 years) compared to young women (18-25 years), elderly women (>60 years) and elderly men (>60 years). The apparent total body clearance of the *S*-enantiomer, did not differ between subject groups.

A study by Fujita *et al* (1990) explored in some detail the hypothesis that the livers of old male rats are functionally feminised. Their data enabled them to propose that senescence-associated feminisation of the drug metabolising capacity of male rat liver may be partly attributable to the decrease in testosterone levels in old age and partly attributable to the loss of neonatal imprinting.

Female rats appear to be more sensitive to the toxic effects of phosphorothioates compared to males, for example the oral LD50 for parathion is 3.6mg/kg and 13mg/kg for females and males, respectively (Gaines, 1960). A study by Chambers *et al* (1994) set out to elucidate the influence of metabolism on this phenomenon. Their data allowed the authors to suggest that differences between the sexes in capacity to activate and detoxify parathion may have contributed to subtle differences in development of toxicity in their animal model. However, as emphasised earlier, it is recognised that sex differences are more important in the rat than man.

Age related factors also appear to influence the development of neurotoxic effects following exposure to certain phosphorothioates. Chicks do not develop OPIDN when treated with doses of phosphorothioate known to be effective in the adult hen (Johnson and Barnes, 1970). However, the resistance of chicks is not absolute since a marginal neuropathic response was obtained either with repeated doses (Johnson and Barnes, 1970) or by substantially increasing the single dose. The clinical features of OPIDN were different in chicks from those seen in adult hens because young animals displayed a spastic gait and not the classical flaccid paralysis. These effects may be interpreted as differential sensitivity of the spinal cord compared to that of the peripheral nerves, or more reasonably, relate to

the faster growth pattern of peripheral nerves in the young. Furthermore, chicks were seen to recover more quickly from OPIDN. Typically, chickens recovered three weeks post development of OPIDN, whilst adult animals took in excess of seven weeks (Lotti, unpublished results). Children are also relatively resistant to OPIDN (Goldstein *et al*, 1988) and when affected they rapidly recover. Age-related resistance to OPIDN may therefore be most easily explained by the faster growth of peripheral nerves in young animals and/ or with the more efficient repair capabilities of the developing nervous system.

5.4 Environmental factors

As discussed above, the levels and activities of the cytochromes P450 vary from one individual to the next due to physiological, including genetic, factors. Cytochrome P450 enzyme activity can also be influenced by environmental factors, such as exposure to a xenobiotic that inhibits or inactivates it. This will, in fact, mimic the effects of a genetic deficiency in P450 enzyme expression and may be a cause of serious adverse clinical consequence. Exposure to xenobiotics *in vivo* may also increase the activity and/ or level of the cytochromes P450. As an underlying cause of serious adverse drug reactions, P450 induction is generally less important than P450 inhibition, because the latter can cause a rapid and profound increase in blood levels of a drug which may then cause symptoms of drug overdose. However, drug interactions have been noted following induction of the cytochromes P450 since one xenobiotic can stimulate the metabolism of a second and thereby decrease its effect. Inhibition and induction/ activation of the cytochromes P450 will be discussed in the following chapter.

Inhibition may be the result of competition of two substrates for the same enzyme but it can also be mechanism-based, involving what are termed "suicide" substrates as they require conversion by the enzyme to a reactive species. Non-competitive inhibition occurs when a xenobiotic binds to the enzyme in such a way as to alter the activity of the enzyme towards its substrate. The requirement for catalysis adds an extra degree of potential selectivity compared with reversible inhibitors, which rely solely on binding (Rando, 1984). A further advantage of mechanism-based inactivators *in vivo* is that the enzyme inhibition persists after the free compound has been cleared, in contrast to a reversible inhibitor, which must be present continuously. Table 5.1 gives examples of substrates and inhibitors of the major human liver microsomal P450 isoforms involved in xenobiotic transformation.

The ability of the macrolide antibiotic erythromycin to influence the pharmacokinetics of other drugs was exemplified by a report (Olkola *et al*, 1993) of the "undesirably severe and excessively long-lasting hypnotic effects" seen when young adults were treated with erythromycin prior to oral administration of the usually short-acting hypnotic agent, midazolam. Both erythromycin (Watkins *et al*, 1985) and midazolam (Kronbach *et al*, 1989) are metabolised by CYP3A and would therefore compete for the active site of the enzyme. Similarly, toxic responses have been seen with the immunosuppressant cyclosporine A and the antihistamine terfenadine following pre-treatment with ketoconazole, an antimycotic agent (Watkins, 1994). All three drugs are metabolised by CYP3A.

Compounds containing olefinic functionalities (eg the CYP3A inhibitor, gestodene) destroy the haem prosthetic group of cytochrome P450

Table 5.1 Examples of substrates and inhibitors of the major human liver microsomal P450 isoforms involved in xenobiotic transformation.

CYP involved	Substrates	Inhibitors
CYP1A2	Acetaminophen Caffeine Estradiol Ethoxyresorufin Phenacetin Theophylline	Furafylline ^a α-Naphthoflavone
CYP2A6	Butadiene Coumarin Nicotine	Diethyldithiocarbamate ^a 8 - Methoxypsoralen ^a
CYP2B6	Cyclophosphamide Ifosphamide	Metyrapone Orphenadrine ^a
CYP2C8	Carbamazepine	Quercetin
CYP2C9	Diclofenac Phenytoin Tolbutamide Warfarin	Sulphaphenazole
CYP2C18/ 19	Hexobarbital Lansoprazole S-Mephenytoin Omeprazole Proguanil Propranolol	Tranylcypromine
CYP2D6	Debrisoquine Flecainide Imipramine Mexilitine Paroxetine Sparteine	Ajmalicine Quinidine Yohimbine
CYP2E1	Alcohols Aniline Benzene Chlorzoxazone p-Nitrophenol Styrene	Diethyldithiocarbamate ^a Dimethyl sulphoxide Disulfiram 4-Methylpyrazole
CYP3A4/ 5	see table 4.1	Gestodene ^a Ketoconazole Naringenin Troleandomycin ^a Quercetin

^a Mechanism-based inhibitor

From A. Parkinson (1996). Toxicologic Pathology 24: 45-57.

following oxidative activation of the xenobiotic (Murray and Reidy, 1990), whereas piperonyl butoxide, an insecticide synergist, forms a stable inhibitory complex with the haem of P450, thereby inhibiting the enzyme. Some of the currently available anti-HIV drugs, which are marketed to inhibit the HIV protease enzyme, have been shown to be substrates for CYP3A and therefore can competitively interact with other xenobiotics metabolised by this isoform (Lipsky, 1996).

Studies with both microsomes (Morelli and Nakatsugawa, 1978) and purified enzymes (Halpert *et al*, 1980) and phosphorothioates such as parathion that contain a P=S moiety have demonstrated that, during oxidative desulphuration, the released sulphur exists as a highly reactive molecule which then binds to P450, inactivating the enzyme. This binding of reactive sulphur is accompanied by loss of P450 as detected by measurement of the dithionite-reduced CO complex as well as loss of enzyme activity (Neal, 1985). A more recent study (Butler and Murray, 1993) of rat hepatic microsomal fractions has shown that the NADPH-mediated conversion of parathion to paraoxon occurs with concomitant inhibition and inactivation of the major constitutive P450s, 3A2 and 2C11.

Exposure of humans to phosphorothioate pesticides such as parathion therefore raises the concern that the metabolic capacity of the individual may be compromised and that untoward effects from subsequent drug therapy to alleviate the anticholinergic effects of intoxication may be experienced. In this context there has been a report that the tranquilliser promazine, given as supportive therapy following parathion intoxication, produced hypotension, convulsions, apnea and then death (Arterberry *et al*, 1962). Because these are symptoms of promazine overdosage, and not of parathion toxicity, it seems that phosphorothioate exposure can, in some

instances, produce a significant decrease in the metabolic capacity of certain human cytochromes P450.

Dietary components may also influence the cytochromes P450. For example, the consumption of a single glass of grapefruit juice can cause up to a fivefold increase in the area under the curve of nifedipine or felodipine, CYP3A substrates (Bailey *et al*, 1991). This was believed to be due to the bioflavonoid aglycone naringenin (4,5,7-trihydroxyflavanone), the so-called "bitter principle" of this beverage. However, recent studies (Edwards and Bernier, 1996) have suggested that other compounds, present in both grapefruit and Seville orange juice, may add to this inhibitory effect. Quercetin, a bioflavonoid aglycone present in high concentrations in some fruits and vegetables, but not grapefruit juice, also appears to be a potent CYP3A inhibitor *in vitro* (Kuhnau, 1976).

Cigarette smoke condensate has also been shown to contain strong inhibitors, as well as substrates, of CYP1A (Shimada and Guengerich, 1991). Foodstuffs such as watercress and green tea also contain P450 inhibitors (Chung *et al*, 1992).

Induction is the process whereby enzyme activity increases through the action of a xenobiotic, thereby increasing the amount of enzyme present in the cell. This action was first described during early studies of the metabolism of carcinogens and barbituates. Studies included those of Conney *et al* (1956) who demonstrated that as little as 0.1mg of 3-methylcholanthrene could triple the demethylase activity of mature male rats, and this action could be repeated using other polycyclic aromatic hydrocarbons (PAHs). Independently, Remmer (1958) showed that the metabolism of various xenobiotics was increased on barbiturate

administration, and that phenobarbital was the classical model compound. It is now known that five classes of inducers exist for the cytochrome P450 isoforms and these are given in table 5.2.

Enhanced P450 enzyme activity can result from:

(i) Gene duplication leading to overexpression of a P450 enzyme, eg demonstrated in individuals classified as "ultrarapid hydroxylators". The molecular basis for two individuals who were ultrarapid hydroxylators of the CYP2D6 substrates nortriptyline and clomipramine was shown to be an *Xba*I 42kb fragment which contained two different functionally active *CYP2D6* genes in the *CYP2D* locus causing extra enzyme to be expressed (Bertilsson *et al*, 1993).

(ii) Exposure to environmental factors, such as xenobiotics, that induce the synthesis of cytochrome P450. This is the most common mechanism by which activity is increased.

(iii) Activation of a preexisting enzyme by a xenobiotic. Although activation of the cytochromes P450 has been documented *in vitro*, it appears to occur *in vivo* only under special circumstances.

In general terms, increased xenobiotic metabolism can be the result of several effects including increased *de novo* synthesis of drug metabolising enzymes, increased stability of mRNA coding for the enzyme and reduced degradation of the enzyme. PAHs induce CYP1A1 by transcription activation via the Ah receptor, and CYP1A2 via stabilising already transcribed mRNA, enabling more protein to be translated from it. Phenobarbital induction of CYP2B and CYP3A1/2 is by transcriptional activation, similar to CYP1A1 induction by PAH but no Ah receptor equivalent has yet been identified. CYP3A1/2 induction by troleandomycin and CYP2E1 induction by acetone is through stabilisation (reduced degradation) of the specific cytochrome P450 protein (Koop and

Table 5.2

Examples and characteristics of five classes of inducers of rat liver microsomal cytochromes P450.

Representative inducers	Major inducible P450 enzyme(s)	Characteristic enzymatic reaction
3-Methylcholanthrene β -Naphthoflavone TCDD Planar PCBs	CYP1A1 and CYP1A2	7-Ethoxyresorufin O-dealkylation Benzo[α]pyrene 3-hydroxylation Caffeine N-3-demethylation
Phenobarbital Antioxidants Antihistamines Nonplanar PCBs	CYP2B1 and CYP2B2	Testosterone 16 β -hydroxylation 7-Pentoxyresorufin O-dealkylation 7-Benzyloxyresorufin O-dealkylation
PCN* Dexamethasone Troleandomycin	CYP3A1 and CYP3A2	Testosterone 2 β -, 6 β -, 15 β -hydroxylation Erythromycin N-demethylation Nifedipine N-oxidation
Isoniazid Pyrazole Ethanol Acetone	CYP2E1	Chlorzoxazone 6-hydroxylation <i>p</i> -Nitrophenol hydroxylation
Clofibric acid and other peroxisome proliferators	CYP4A1-3	Lauric acid 12-hydroxylation

* PCN is pregnenolone-16 α -carbonitrile

Compiled from A. Parkinson (1996). Toxic Pathol 24: 45-57

Tierney, 1990). Glucocorticoid (eg dexamethasone) and clofibrate induction of P450s 3A and 4A, respectively, is via a receptor mediated transcription stimulation pathway and stabilisation of the newly synthesised protein, similar to that of the Ah induction pathway (Waxman and Azaroff 1992).

The induction of CYP3A by macrolide antibiotics, such as troleandomycin and erythromycin, primarily involves their transformation to metabolites that bind tightly to the haem moiety of P450, thereby inhibiting the enzyme. The induction of CYP3A by macrolide antibiotics, as described above, is often masked by their capacity to function as mechanism-based inhibitors. Similarly, the enzyme-inducing effects of imidazole antifungal agents, such as ketoconazole, are often masked by their ability to bind and inhibit CYP3A (as well as other cytochromes P450). This phenomenon was demonstrated by Thompson *et al* (1988) who found inhibition of hepatic microsomal ethoxyresorufin O-deethylation (CYP1A) and aldrin epoxidation (CYP3A and CYP2B) activity 2hr after oral dosing of rats with ketoconazole (50mg/ kg and 100mg/ kg) which was followed by induction 1day and 7days later.

As discussed earlier, CYP3A is highly inducible by treatment with many medications in common use (e.g. dexamethasone, phenobarbital, rifampicin), and this appears to explain why patients administered with these drugs require increased dosing of cyclosporine A (a CYP3A substrate) (Pichard *et al*, 1990).

In general, highly chlorinated compounds are resistant to biotransformation and cause prolonged induction of the cytochromes P450 and other enzymes. For example, CYP3A also appears to be inducible, at least in rodents, by many environmental contaminants, including

polyhalogenated biphenyls (PCBs and PBBs) (Dannan *et al*, 1983). In the human population, workers occupationally exposed to the organochlorine pesticides lindane or DDT exhibited shortened plasma half lives of antipyrine and phenylbutazone (Conney *et al*, 1971; Kolmodin-Hedman, 1973). In the study conducted by Conney *et al*, the DDT workers were shown to have an elevated 6 β -hydroxy cortisol excretion, a marker of enzyme induction.

Induction of other enzyme systems has also been demonstrated. McCracken *et al* (1992) investigated the effects of phenobarbital, clofibrate and β -naphthoflavone (a PAH type inducer) on carboxylesterase, paraoxonase and arylesterase activity of rat liver, lung and skin. They found that β -naphthoflavone and clofibrate did not induce carboxylesterase, paraoxonase or arylesterase activity, while phenobarbital induced carboxylesterase in the liver and skin, paraoxonase in the liver only, and arylesterase in the lung only.

Phase 2 enzymes can also be enhanced by the classical inducers. Rat liver glutathione-S-transferases are induced by both phenobarbital and 3-methylcholanthrene, for example Pickett *et al* (1984) reported elevated α and μ class glutathione-S-transferases in rat liver on treatment with these inducers, while Primiano *et al* (1992) showed increased glutathione transferase expression in the rat using imidazoles as inducers.

Evaluation of the effects of chemical inhibitors and inducers on the cytochromes P450 may be used *in vitro* to help elucidate the catalytic specificity of the various isoforms in a particular metabolic reaction. Advantages of using chemical inhibitors as a tool in metabolism studies include the fact that they are commercially available (or can be easily

synthesised), may be used with intact cells and also *in vivo*, even with human subjects in many cases eg sulphaphenazole (CYP2C), furafylline (CYP1A2). However, chemical inhibitors of the cytochromes P450 must be used with caution because most of them can inhibit more than one isoform.

The classical inducers described above and in table 5.2 may be used as a tool to indicate the specificity of the cytochromes P450 in a particular metabolic pathway. In order to achieve this a range of inducers may be administered to groups of rodents *in vivo*, followed by evaluation of changes in the levels of microsomal cytochromes P450 (and other enzymes) *in vitro*. The inhibitors and inducers used in this thesis to indicate the enzymes responsible for parathion metabolism is shown schematically in figure 5.1.

5.5 The route of absorption

Most phosphorothioate pesticides are not ionised and are relatively lipophilic, they therefore require metabolic transformation prior to excretion. They may be easily taken up by a variety of natural routes including ingestion, inhalation and dermal absorption. Most exposures involve more than one route, and in these cases it is the character of the chemical involved that determines which of the routes will be of practical importance. Their low vapour pressures under normal useage means that they do not often enter the body by inhalation and the skin is the usual route of exposure.

Following ingestion, the absorbed phosphorothioate will gain access to the venous circulation via the liver where it will undergo first pass

An outline of the enzymes which may be involved in parathion metabolism in the rat and man.

LIVER ENZYMES (RAT)	
P450 isoforms	Esterases
CYP1A1/2	A-esterase
CYP2B1/2	B-esterase
CYP2C13/11	
CYP2D1	
CYP2E1	
CYP3A1/2	

LIVER ENZYMES (HUMAN)	
P450 isoforms	Esterases
CYP1A1/2	A-esterase
CYP2B6	B-esterase
CYP2C8	
CYP2C9	
CYP2D6	
CYP2E1	
CYP3A4/ 5	

Model Substrate	Inhibitor/ Substrate	Inducer
Ethoxyresorufin	α -NF	β -NF
Pentoxyresorufin	Metyrapone	Phenobarbital
--	Tolbutamide*	--
--	Sulphaphenazole	
--	Quinidine	--
<i>p</i> -Nitrophenol	DEDC	Ethanol
Nifedipine	Naringenin, Quercetin, Ketoconazole, Troleandomycin	Dexamethasone, Phenobarbital
Paraoxon	EDTA	--
Phenylvalerate	--	Phenobarbital

* substrate

-- not determined



Objective
1) to determine the human CYP(s) involved in parathion metabolism
2) to indicate the role of metabolism in individual susceptibility to organophosphate toxicity

detoxification and/ or activation, depending on the compound. The uptake of most phosphorothioate following administration by the oral route is rapid and efficient under test conditions. However, there is evidence of comparatively inefficient absorption in hens administered large doses of very insoluble phosphorothioates with a high relative molecular mass, for example leptophos (phosphonothioic acid, phenyl-, O-(4-bromo-2,5-dichlorophenyl) O-methyl ester). Thus, divided doses may exert a greater toxic effect than the same amount given as a single large dose.

Following inhalation of phosphorothioates in spray formulations or powders, particles of diameter larger than 5-10 μ m are efficiently trapped in the upper respiratory tract where they become impinged on its surface mucous layer and carried upward into the pharynx by ciliary action. Here they are swallowed. Particles of diameter between approximately 1-5 μ m are retained by the alveolar region of the lungs where they are subsequently absorbed. They are cleared from the lungs by way of the macrophages and pulmonary blood supply. Absorption from the alveoli tends to be rapid compared with the rate by other portals, because the alveolar membrane is thin and the blood supply abundant.

Many phosphorothioates are effectively absorbed through the skin and this route of absorption is especially important in connection with modern pesticides because many of them were selected as contact poisons. The same chemical properties that make phosphorothioate pesticides able to penetrate the insect cuticle permit their dermal absorption by mammals. However, in practice, only a small portion of the skin may become contaminated and, therefore, only that part will participate in absorption. A compound's solubility and particle size are the main factors

which will influence percutaneous absorption but, in general terms, absorption will be greater for the more lipophilic phosphorothioates.

It is likely that percutaneous absorption will be greater for liquid formulations than for powders, but that powders may adhere longer thereby enhancing an effect, if proper hygiene is not observed. Respiratory exposure was noted to be increased 4-fold when applying dusts compared with dilute spray, and 10-fold when using aerosols of concentrated pesticide. Thus, in the latter case, the respiratory route could be very important when the efficiency of absorption is allowed for.

Other routes such as subcutaneous (sc), intra-peritoneal (ip) and intravenous (iv) injection are more frequently used in laboratory studies of the effects of chemicals and administration by these routes assures that an entire, carefully measured dose can be given without loss. The route of absorption will be a major factor influencing metabolism. Organophosphates administered by the sc, ip and iv routes will enter the peripheral venous circulation directly and distribute into tissues and organs, thus bypassing extensive first-pass hepatic metabolism. On the other hand, if the chemical is delivered orally, it will gain access to the venous circulation via the liver prior to any distribution into the systemic circulation. First-pass metabolism may account for the loss of a large proportion of the parent compound prior to its reaching the systemic circulation where the blood esterases will play a significant detoxification role.

5.6 Extrahepatic metabolism

Although the liver is generally the organ with the highest drug-metabolising activity, the P450s are also present in other tissues, including the small intestine, kidney, brain and lung. In fact, because CYP3A is abundant in the intestinal mucosa, xenobiotic substrates for this subfamily may have poor oral bioavailability due to extensive metabolism at the level of the intestine (Watkins, 1994). Indeed, the small intestine and lung could present the first line of defence against absorbed toxins. Any local biotransformation may be important for the compounds having these tissues as their targets, and may also considerably influence the general metabolism, activity and toxicity of xenobiotics.

Both microsomal and mitochondrial forms of cytochrome P450 have been found in the brain. In addition to the estimation of the total amount of cytochrome P450 in the brain by its carbon monoxide difference spectrum following partial purification, several specific isoforms have been detected by their catalytic activities, by immunohistochemical techniques and the mRNA for some isoforms have been detected by Northern Blotting (Warner and Gustafsson, 1993). Cytochrome P450 levels in the brain are very low, in untreated rats the concentration is approximately 1% - 3% of that found in the liver. The distribution throughout the different brain regions is uneven with the highest levels to be found in the cerebellum and the lowest in the substantia nigra.

Cytochromes P450 are of particular importance in lung since the lung is the main portal of entry for various airborne contaminants. It is now clear that, although the level of cytochrome P450 content and enzyme activities are quite low in lung when compared to those in liver, the lung

cytochrome P450s have the capacity to metabolise a wide variety of xenobiotics (Arinç, 1993). The lung may play an important role in the local activation of phosphorothioates which have the potential to enter the body via aerosols generated occupationally, eg during spraying of wasps and other pests. Phosphorothioates contained in sheep dips may also enter the lung by way of the fine spray generated during the dipping process.

The skin is the main portal of entry for phosphorothioate pesticides and therefore deserves to be discussed in this chapter. Cytochrome P450 levels in the skin, as in all extrahepatic tissues, are low with activities around 0 - 5% of that seen in the liver. Activities are typically lower in human skin compared to rodent (Damen and Mier, 1982). In contrast to the P450s, several studies have shown relatively high esterase activity in the skin (Cheung *et al*, 1985; McCracken *et al*, 1993). In addition, Venkatesh *et al* (1992) reported that the FMO-mediated sulphoxidation of the pesticide phorate in skin microsomes was about four fold higher than that seen in the liver.

Chapter 6

Chapter 6 Experimental aims

6.1 Experimental aims

It has been known for over thirty years that the cytochromes P450 are involved in the activation of phosphorothioate pesticides to the toxic oxon (Neal, 1964). However, until very recently, there was a huge deficit in the literature regarding involvement of the individual P450 isoforms in the reaction. Moreover, the role of detoxification enzymes in phosphorothioate metabolism in man has been poorly investigated, even though it is recognised that a polymorphism exists in the plasma A-esterases which hydrolyse the toxic oxons (Geldmacher-von-Mallinckrodt and Diepgen, 1988).

It is recognised that individuals vary widely in their toxic response to exposure to phosphorothioates, but few reasons have been put forward as to why this should be so. This thesis investigated the hypothesis that variations in toxic response may be influenced by individual differences in the capacity to metabolise phosphorothioates following exposure.

The phosphorothioate parathion was chosen as the main phosphorothioate pesticide to study differences in xenobiotic metabolising potential of human liver microsomes. Parathion was chosen because:-

- a) It has a relatively simple metabolism, and requires oxidative biotransformation to the oxon for toxicity.
- b) It is a typical anti-cholinesterase compound which is not believed to be neurotoxic, and therefore is relatively safe to handle.

c) It has been in use for a number of years and therefore an extensive toxicity data base exists in the literature.

The experimental section of the thesis was essentially in two parts: an animal and a human study. In the first part, male Wistar rat liver P450s and esterases were modulated *in vivo* and *in vitro* in order to elucidate the metabolic pathways involved in parathion metabolism. Next, the human section concentrated on studies with sixteen liver microsomal preparations and commercially available human P450-expressed microsomes. These studies indicated the P450 isoforms and esterases involved in parathion metabolism and assessed variation between individuals in their capacity to carry out the reactions.

The experimental aims of the thesis were therefore:-

- 1) To define the capacity of control microsomes to metabolise parathion to paraoxon and *p*-nitrophenol and to determine the kinetics of the reaction and to characterise control rat liver microsomal activities for the cytochromes P450, FMO and esterases which may be involved in parathion metabolism.
- 2) To investigate the various groups of induced liver microsomes for capacity to metabolise parathion and to determine the efficacy of the induction regimen by measuring the increase in activity of the cytochromes P450 and esterases compared to control.
- 3) To modulate control rat liver microsomes with various P450 inhibitors, *in vitro* in order to indicate the constitutive P450 isoform(s) involved in parathion metabolism.

- 4) To define interindividual variations in capacity of the microsomal panel to metabolise parathion and to determine the kinetics of the reaction and to characterise the panel of human liver microsomes for CYP 1A1/2, 2B6, 2E1, 3A4/5, A-esterase, B-esterase and glutathione-S-transferase activities.
- 5) To correlate the P450 and esterase activities determined for the human liver microsomes with their capacity to produce paraoxon and *p*-nitrophenol from parathion.
- 6) To modulate human liver microsomes with P450 and esterase inhibitors *in vitro* in order to indicate the constitutive P450 isoform(s) and esterases involved in parathion metabolism.
- 7) To compare the capacity of commercially available microsomes from cell lines expressing human P450s 3A4, 3A5, 1A1, 2B6, 2E1, 2C8 and 2C9-Arg₁₄₄ to metabolise parathion.
- 8) To define the way in which a balance between activation and detoxification of parathion could contribute to interindividual differences in circulating/ local levels of oxon and thus toxic effects.
- 9) To use this data to extrapolate to other phosphorothioates.

SECTION II: METHODS

Chapter 7

Chapter 7 General methods

7.1 Chemicals

Unless otherwise specified general laboratory chemicals were obtained from BDH Chemicals Ltd., Dorset, UK; Sigma-Aldrich Company Ltd., Dorset, UK; or Fisons Scientific Equipment, Loughborough, UK. GC and HPLC columns were obtained from Hewlett-Packard Ltd., Cheadle Heath, Cheshire and Fisons Chromatography, Loughborough, UK., respectively.

Parathion and paraoxon were obtained from British Greyhound Chromatography and Allied Chemicals, Birkenhead, Merseyside, UK and *p*-nitrophenol from Sigma-Aldrich Company Ltd. Pure substance M1 (BAY b4759), the major metabolite of nifedipine oxidation, was supplied by A.G. Bayer, Bayer House, Newbury, U.K.

Caution paraoxon is a potent anti-cholinesterase organophosphate. Take great care especially when handling concentrated solutions; refer to COSHH safety procedure.

7.2 Animals

Male Wistar rats were obtained from the Comparative Biology Centre, University of Newcastle upon Tyne. The rats were 8-9 weeks of age, weighed approximately 250g and were housed on wood shavings with a 12 hour light cycle. The animals were fed standard rodent laboratory diet (RM3 expanded. Specialist Diet Services, Witham, Essex.) and allowed free access to water unless otherwise stated.

7.3 Buffer solutions

- 1) *0.1M potassium phosphate (KH_2PO_4)/ 0.1M potassium chloride buffer* Adjust pH to pH 7.4 with sodium hydroxide prior to use.
- 2) *50mM glycine/ 1mM calcium (CaCl_2) buffer* Adjust pH to pH 7.0, 7.4, 8.0, 9.0, 10.0 or 10.5 with sodium hydroxide prior to use.
- 3) *50mM Tris/ 0.2mM EDTA buffer* (pH 8.0 at 37°C) was prepared by adding EDTA to Trizma pre-set crystals (pH 8.0).
- 4) *500mM Tris buffer* (pH 8.5 at 37°C) was prepared from Trizma pre-set crystals (pH 8.5).
- 5) *50mM glycine buffer* Adjust pH to pH 8.5 with sodium hydroxide.
- 6) *10mM potassium phosphate (KH_2PO_4) buffer* Adjust pH to pH 7.4 with sodium hydroxide.
- 7) *100mM potassium phosphate (KH_2PO_4)/ 1mM ascorbic acid/ 5mM magnesium chloride buffer* Adjust pH to pH 6.8 with sodium hydroxide.
- 8) *2M Tris buffer* (pH 9.0 at 37°C) was prepared from Trizma pre-set crystals (pH 8.5).
- 9) *50mM Tris/ 1mM calcium buffer* (pH 7.4 at 37°C) was prepared by adding CaCl_2 to Trizma pre-set crystals (pH 7.4).
- 10) *100mM potassium phosphate (KH_2PO_4) buffer* Adjust pH to pH 6.5 with sodium hydroxide.

7.4 Preparation of rat and human liver subcellular fractions

All steps were carried out on ice or at 4°C.

Human or rat liver homogenates (10%, w/v) were prepared in ice cold potassium chloride/ phosphate buffer (0.1M KCl/ 0.1M KH₂PO₄, pH 7.4) using a Polytron cell homogeniser (Kinematica AG, Littau-Luzern, Switzerland). Homogenates were centrifuged in a Mistrall refrigerated centrifuge at 1 500g for 5 minutes at 4°C to remove tissue debris. The supernatants were further centrifuged at 12 500g for 10 minutes to remove cellular nuclei and mitochondria. The post-mitochondrial fractions were then centrifuged in a Sorvall ultra-centrifuge at 100 000g for one hour and ten minutes at 4°C.

The supernatants (cytosol) were removed and the surface of the resulting microsomal pellet washed carefully with potassium chloride/ phosphate buffer. Microsomal pellets were then resuspended in the same buffer using a glass to glass homogeniser. The suspensions were vortexed gently to free the microsomes of trapped debris and then re-centrifuged at 100 000g for one hour. The microsomal pellets were then resuspended in 2ml potassium chloride/ phosphate buffer using a hand held glass to glass homogeniser. Samples were stored in aliquots at -70°C prior to analysis.

7.5 Determination of the protein concentration of subcellular fractions

The method used in the study was an adaptation by Peterson (1977) of the original Folin-Ciocalteu reagent method as given by Lowry and co-workers in 1951 for estimation of total protein. The original procedure was

modified to provide a method which was simple, rapid, objective and more generally applicable. SDS (sodium dodecyl sulphate) was added to alleviate possible non-ionic and cationic detergent and lipid interferences and to provide mild conditions for rapid denaturation of membrane and proteolipid proteins.

Reagents

Stock Reagents

1) Copper-Tartrate-Carbonate (CTC)

10g sodium carbonate -----> 100ml dist. H₂O

Add 100mg CuSO₄. 5H₂O and 200 mg KNa tartrate. 4H₂O

2) 10% (w/v) sodium dodecyl sulphate (SDS)

10g SDS -----> 100ml dist. H₂O

3) 0.8N sodium hydroxide

32g -----> 1litre dist. H₂O

4) Folin-Ciocalteu phenol solution

Commercially available from BDH

Working Solutions

1) Bovine Serum Albumin ("Precimat" protein standard)

Purchased from Boehringer as a 6g/100ml solution.

1: 100 dilution was prepared in dist. H₂O

(100μl -----> 10ml dist. H₂O).

This working solution was 600μg protein/ml.

2) Reagent A

Mix equal volumes of stock CTC, NaOH, SDS and dist. H₂O.

The solution is stable for 1-2 weeks in the dark at 20°C

(discard solution when a dark precipitate begins to appear).

3) Reagent B

20ml Folin-Ciocalteu phenol reagent + 100ml dist. H₂O

The solution is stable for several months in the dark at 20°C.

Procedure

A known aliquot of microsomal suspension or cytosol was made up to a total volume of 1ml with distilled water. 1ml reagent A was added to each tube, the tubes mixed and allowed to stand for 10min at room temperature. 0.5ml reagent B was then added and the tubes mixed immediately. After 30min the samples were put in quartz cuvettes of 1cm pathlength and their absorbances read using a Kontron 930 spectrophotometer set at 750nm. Samples were read against a reagent blank placed in the reference position.

A standard curve (0 - 72µg protein) was prepared in 1ml aliquotes of distilled water and then treated in the same manner as the samples. The standard curve was best fitted to a quadratic equation. Samples were quantitated by comparison to the standard curve using a small programmable calculator, or directly by the Kontron 930 spectrophotometer.

7.6 Spectral determination of the P450 content of rat liver microsomes

Cytochrome P450 is a haemoprotein and use is made of the fact that when the haem iron is reduced and complexed with carbon monoxide, a characteristic absorption spectrum results. The reduced carbon, monoxide difference spectrum of cytochrome P450 absorbs maximally at around 450nm and the extinction coefficient for the wavelength couple 450nm - 490nm has been accurately determined to be 91mM⁻¹ cm⁻¹, thus allowing

quantitative determination of this haemoprotein. The method used was an adaptation of that described by Omura and Sato (1964).

Procedure

Liver microsome preparations were diluted in KCl/ phosphate buffer (pH 7.4) to approximately 1mg protein/ ml. A 2ml aliquot of the diluted sample was added to matched sample and reference quartz cuvettes and a baseline recorded between 400nm and 500nm. A Kontron 930 double-beamed spectrophotometer was employed. A few grains of solid sodium dithionite was added to each cuvette and then mixed by gentle inversion. The sample cuvette was then placed in an extraction hood and bubbled gently (approximately 1 bubble/ sec) with carbon monoxide for approximately 1min. The spectrum was then re-scanned from 400nm to 500nm. The absorbance difference between 450nm and 490nm was then calculated.

Using Beer's Law and assuming a pathlength of 1cm, a difference spectrum of 0.03, a cuvette protein concentration of 10mg/ ml which had been diluted 1:20, the cytochrome P450 concentration is given by:

$$\frac{0.03 \times 20 \times 1000}{91 \times 10} = 0.659 \text{ nmol P450/ mg protein}$$

7.7 Statistical methods

Unless otherwise stated statistical analysis was by ANOVA followed by Newman-Keuls post test for multiple comparisons which was used to identify which of the means actually differed.

Animal data were expressed as the mean \pm standard error of the mean (sem). Data derived from human studies were summarised as enzyme activity ranges and medians since the data could not be considered to be homogenous.

In the human studies, the correlation coefficient (r) was calculated by least squares linear regression fit and from this values of t were determined:

$$t = \frac{r \sqrt{(n - 2)}}{\sqrt{(1 - r^2)}}$$

The calculated value of t was compared with the tabulated value at $p < 0.05$ or less, using a two-tailed test and (n-2) degrees of freedom. The data were also analysed by non-parametric statistics, the Spearman rank correlation coefficient (Rho) using the Statworks+™ application for the Apple Macintosh. Values for Rho were compared with the tabulated values at $p < 0.05$ or less, using a two-tailed test.

Chapter 8

8.1. Measurement of the activity of cytochromes P450 1A and 2B

Ethoxyresorufin and pentoxyresorufin can be O-dealkylated by cytochromes P450 1A1/2 and 2B1/2, respectively, in the presence of NADPH and oxygen to produce the common metabolite resorufin. These two substrates are often used as highly sensitive markers for the aforementioned P450 isoforms. The continuous monitoring fluorimetric method used in this study was based on that of Burke and Mayer (1974).

Reagents

Ethoxyresorufin, pentoxyresorufin and resorufin were purchased from Sigma-Aldrich, Dorset, UK. It was necessary to protect all three chemicals from the light. Stock solutions (2mM) of resorufin, ethoxyresorufin and pentoxyresorufin were prepared in DMSO. Resorufin was then diluted to a working concentration of 0.01mM with DMSO. The incubation medium was 10mM phosphate buffer, pH 7.4 (chapter 7). NADPH (1.2 mg/ml) was freshly prepared in phosphate buffer and kept on ice.

The influence of protein, time and substrate concentrations

The ethoxy- and pentoxyresorufin O-dealkylase activities were determined at saturating substrate concentration (2 μ M) to ensure that V_{max} rates were measured. Under these conditions the rate of ethoxyresorufin O-dealkylation was linear up to 0.2mg microsomal protein/ incubation. The initial rate of reaction was assessed and was visually judged to be linear with time.

Procedure

A Perkin-Elmer LS-5 fluorimeter fitted with a water jacketed cell holder set to 37°C and connected to an Amstrad computer with a kinetics package (ELMER) was used. The rate of formation of resorufin was measured with the following fluorimeter parameters: excitation wavelength 530nm, emission wavelength 585nm, excitation and emission slit widths 10nm. The best-fit for the reaction (positive slope) was determined by the kinetics package.

Liver microsomes were equilibrated to 37°C in a microcuvette with NADPH (0.6mg) and phosphate buffer to a final volume of 1ml. The reaction was started on addition of 2 nmoles (1µl) of substrate, to give a final concentration of 2µM, and the reaction followed for up to 5 minutes at 37°C. Analysis was carried out in duplicate.

Quantitation of the reaction

Quantitation of the reaction and determination of quenching of the fluorescence by protein was carried out by measuring the fluorescence produced by 10 pmoles (1µl) of authentic resorufin in 1ml phosphate buffer containing microsomal protein. This value was used to calculate the enzyme activity which was expressed as pmol resorufin produced/ min/ mg microsomal protein.

$$\begin{aligned} \text{pmol resorufin produced/ min/ mg microsomal protein} = \\ \frac{\text{Slope for the reaction (fluorescence units change/min)}}{\text{fluorescence units produced by 1pmol resorufin}} \\ \times \text{mg protein/incubation} \end{aligned}$$

8.2 Measurement of the activity of cytochrome P450 2E1

The hydroxylation of *p*-nitrophenol to 4-nitrocatechol has been used as a marker reaction for cytochrome P450 2E1 activity in both rat and human liver microsomes. The method used in this study for the detection of 4-nitrocatechol was based on that of Tassaneeyakul *et al*, 1993.

Reagents

100mM potassium phosphate/ 1mM ascorbic acid/ 5mM magnesium chloride buffer, pH 6.8 (chapter 7). Stock solutions of *p*-nitrophenol (10mM) and 4-nitrocatechol (10mM) were prepared in buffer and diluted 1:10 and 1:100, respectively, before use. *p*-Nitrophenol solutions were freshly prepared prior to each assay and stored on ice in the dark. A stock solution of salicylamide (8mg/ ml) was prepared in methanol and diluted 1:100 with buffer prior to use. NADPH (1.2 mg/ml) was freshly prepared in phosphate buffer and kept on ice.

The influence of protein, time and substrate concentration

Tassaneeyakul *et al* (1993) reported that human liver microsomes produced 4-nitrocatechol with a K_m of 26.3 μ M, therefore this study used a *p*-nitrophenol concentration of 200 μ M to ensure that V_{max} rates were measured. Under these conditions rates of 4-nitrocatechol formation were linear with time and protein concentration to 30 minutes and 0.5mg/ incubation, respectively.

Procedure

A Kontron HPLC system fitted with u.v./ visible detector set at 250nm was used to measure the absorbance of the metabolites. A reverse-phase separation was employed using a 5 μ Spherisorb ODS1 column (25cm x

4.6mm, Fisons Chromatography, Loughborough, UK) and μ Bondapak C18 precolumn (Waters Chromatography, Hertfordshire, UK). The mobile phase comprised of 40% methanol/ 60% phosphoric acid (0.07%, w/v, pH 3.0) at a flow rate of 1ml/ minute. Using these conditions the retention times for 4-nitrocatechol, salicylamide (internal standard) and *p*-nitrophenol were 24.6, 28.5 and 33.6 minutes, respectively.

Liver microsomes were equilibrated to 37°C in glass test tubes with NADPH (0.3mg) and buffer to a final volume of 0.5ml. Analysis was carried out in triplicate. The reaction was started on addition of 100nmol (100 μ l) of substrate, to give a final concentration of 200 μ M. After a suitable period the reaction was terminated by the addition of 0.6M perchloric acid (0.25ml). Salicylamide (50 μ l) was added to serve as the internal standard and the tubes mixed well. The tubes were then centrifuged for 15 minutes at 500g to pellet the denatured protein. Approximately 0.25ml of each clear supernatant was subsequently removed to glass vials and 80 μ l aliquots injected onto the HPLC system.

Quantitation of the reaction

A standard curve was constructed for 4-nitrocatechol (5 μ M - 40 μ M) in the presence of boiled microsomes (0.5mg protein) and treated in exactly the same manner as the samples. The amount of 4-nitrocatechol produced by the microsomal samples was quantitated by comparison to the standard curve. Enzyme activity was expressed as nmol 4-nitrocatechol produced/ min/ mg microsomal protein.

Several research groups (Iribarne *et al*, 1996; Niwa *et al*, 1995; Shimada *et al*, 1997) have used nifedipine oxidation as a marker reaction for P450 3A activity in rat and human microsomes, as well as other species. This sensitive, efficient, capillary gas chromatographic (GC) method for the detection of nifedipine and its primary metabolite, M1, was based on that of Schmid *et al*, 1988.

Reagents

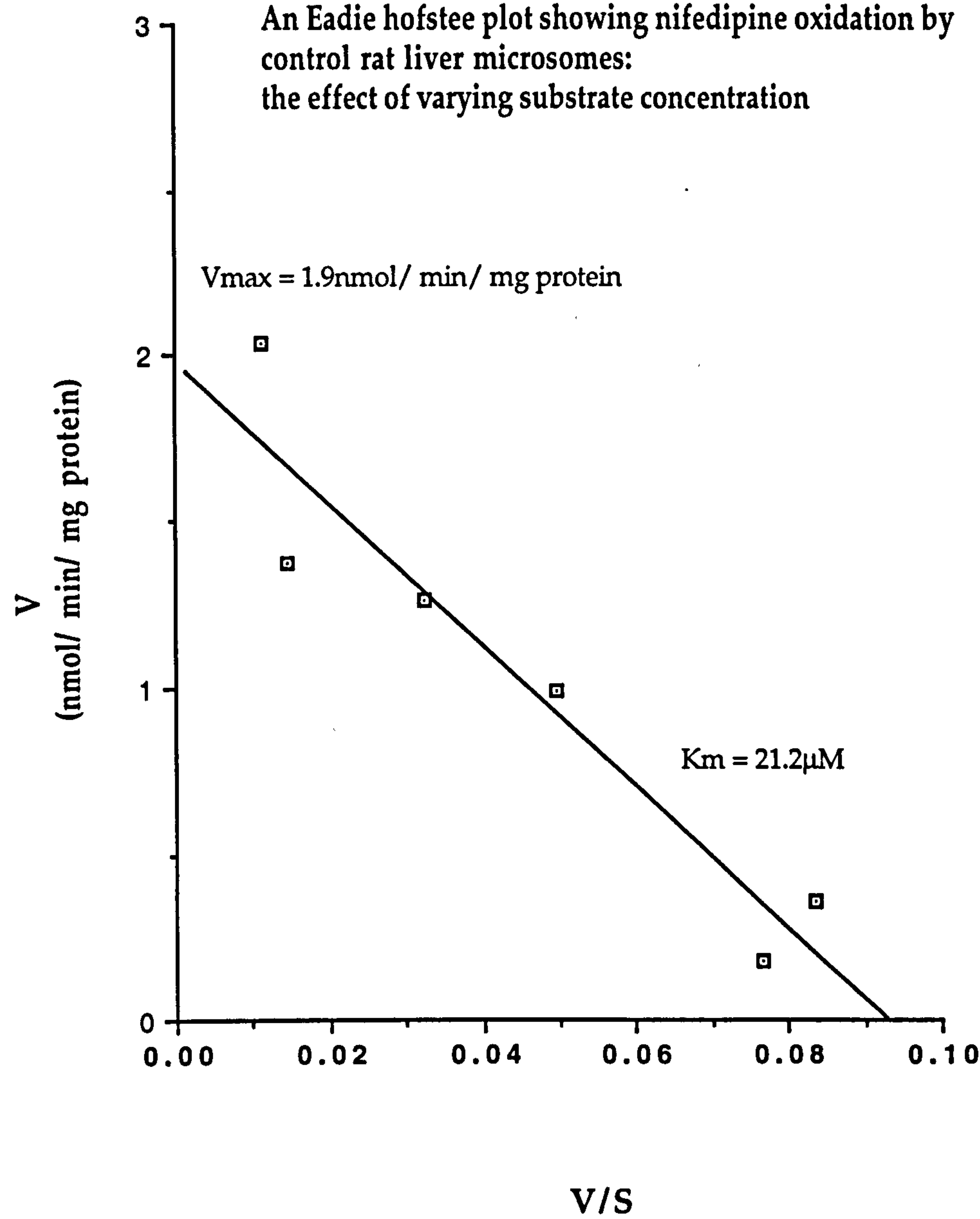
0.1M potassium phosphate/ 0.1M potassium chloride (pH 7.4) and 2M Tris buffers (pH 9.0) buffers, (chapter 7). Stock solutions of nifedipine (25mM) and nitrendipine (0.50mg/ ml, internal standard) were prepared under a sodium lamp in methanol and stored in amber bottles. Stock nitrendipine was diluted 1:20 in water for use. NADPH (6mg/ ml) was freshly prepared in phosphate buffer prior to each assay and kept on ice.

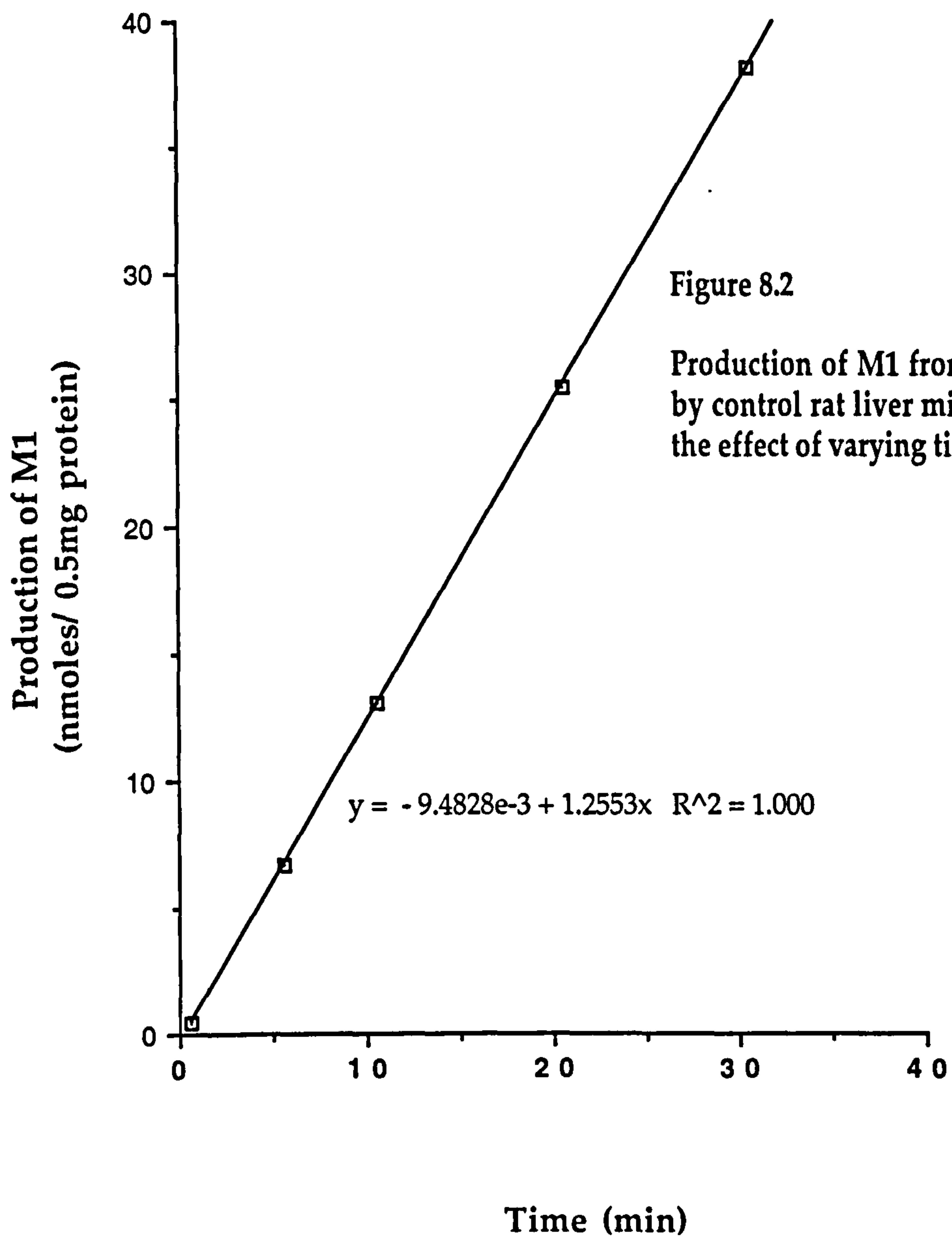
The influence of protein, time and substrate concentration

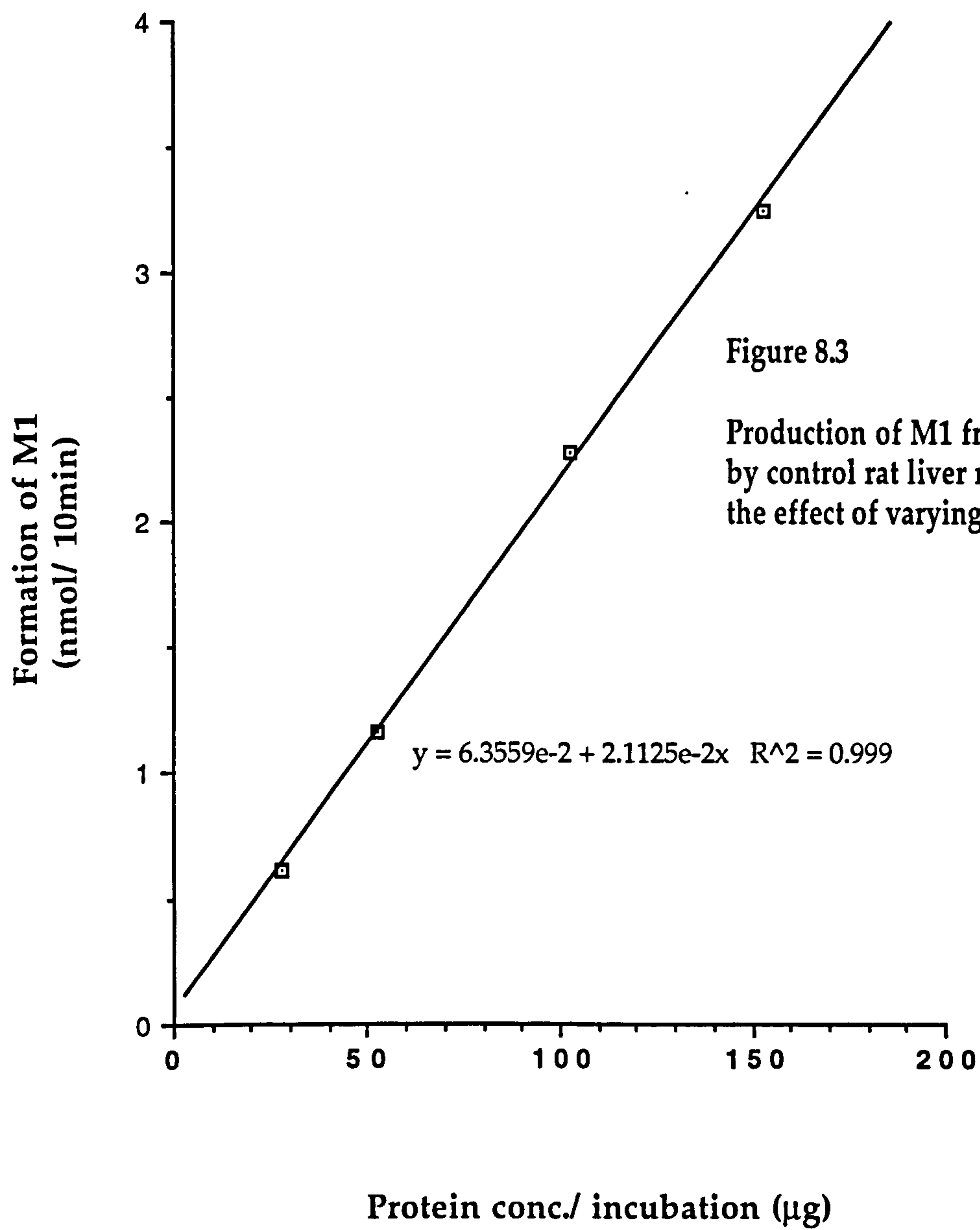
The apparent V_{max} , 1.9 nmol/ min/ mg protein, and apparent K_m , 21.2 μ M, for M1 formation by control rat liver microsomes (n=1) was determined using an Eadie Hofstee plot (figure 8.1) based upon data from a range of nifedipine concentrations (2-200 μ M) in which conditions of linearity with respect to time and protein concentration were employed. This relates to the reported K_m of 6-27 μ M for nifedipine oxidation by human liver microsomes (Guengerich *et al*, 1986). The present study used a saturating substrate concentration of 100 μ M to ensure V_{max} rates. Under these conditions rates of M1 formation were linear with time (figure 8.2) and protein concentration (figure 8.3) to 30 minutes and 1.2mg/ incubation, respectively.

Figure 8.1

An Eadie hofstee plot showing nifedipine oxidation by control rat liver microsomes: the effect of varying substrate concentration







Procedure

A Hewlett Packard (HP 5890A) gas chromatograph (GC) equipped with an unheated on-column injector and a ^{63}Ni electron capture detector (ECD) was employed. Injection (0.2 μl) was achieved directly into a wide-bore HP-1 capillary column (30cm x 0.53mm, 2.65 μm film thickness) which was connected by a dead volume butt connector to a capillary HP-1 column (25m x 0.25mm, 0.25 μm film thickness). The detector temperature was 300 $^{\circ}\text{C}$ and the oven temperature was maintained at 240 $^{\circ}\text{C}$. Carrier gas was hydrogen (1ml/ min) with a nitrogen make-up flow rate of 40ml/ min. A suitably programmed integrator (HP3396A) was used to process the chromatographic signals. Under these conditions the retention times obtained for M1, an unknown metabolite, nifedipine and nitrendipine were 3.3, 4.0, 5.6 and 7.4 minutes, respectively (appendix V).

Microsomal protein was equilibrated to 37 $^{\circ}\text{C}$ in amber glass tubes with NADPH (0.6mg) and phosphate buffer (pH 7.4) to a final volume of 1.0ml. Incubations were carried out in a room fitted with a sodium lamp to exclude daylight in order to prevent decay of nifedipine. Analysis was carried out in triplicate. A "buffer blank" (as above but no protein) was carried out in parallel to ascertain non-enzymatic formation of M1. The reaction was started by addition of 100nmol (4 μl) of nifedipine (25mM), to give a final substrate concentration of 100 μM and methanol concentration of 0.4% (v/v).

After a suitable period the reaction was terminated by addition of 400 μl Tris buffer (pH 9.0). The bottles were capped, vortexed and immediately placed on ice. Nitrendipine (100 μl) was then added to each to serve as the internal standard. The mixture was extracted with 2ml toluene (rotary mixer for 30 minutes) followed by centrifugation for 5min x 1 000g. The

upper organic layer (~1ml) was removed to clean GC vials for detection of nifedipine, nitrendipine (I.S.) and the major metabolite, M1.

Quantitation of the reaction

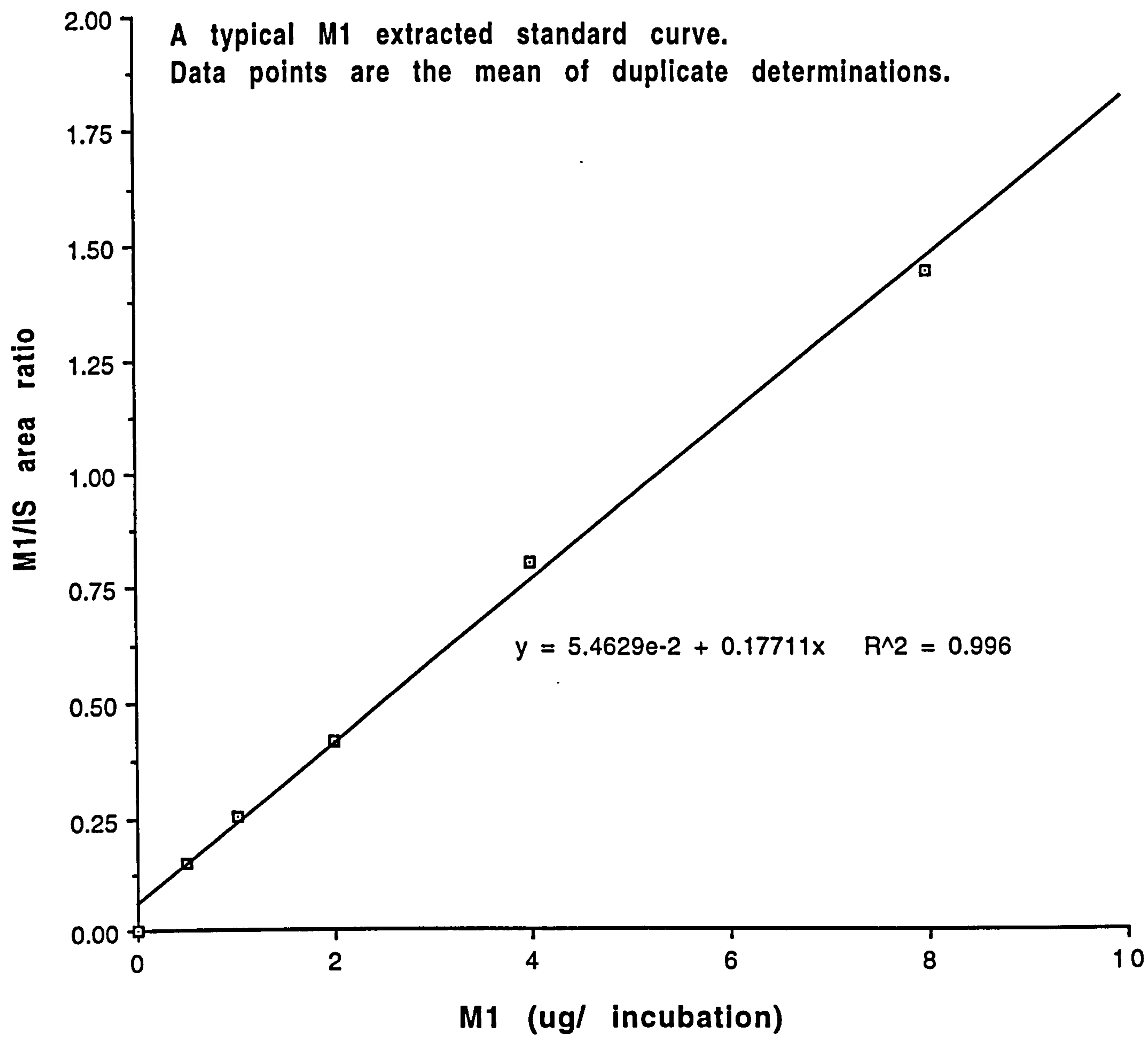
A standard curve (figure 8.4) was constructed for authentic M1 (0.5 - 8µg/incubation, i.e. 1.38 - 22.1nmol/ incubation) in the presence of boiled microsomes (0.5mg protein) and treated in exactly the same manner as the samples. Standards were expressed as area M1/ area internal standard ratios. The amount of M1 produced by the microsomal samples was quantitated by comparison to the standard curve. Enzyme activity was expressed as nmol M1 produced/ min/ mg microsomal protein.

8.4 Method for the study of parathion metabolism

Parathion metabolism has been mostly studied in rodent liver microsomal fractions (Sultatos and Murphy, 1983; Ma and Chambers, 1995), although a few investigators (Nakatsugawa *et al*, 1980) have used hepatocytes where microsomal and cytosolic enzymes function in parallel within the intact cell. Liver metabolism was studied since this organ is classically the most active catalytically and used microsomal fractions, rather than hepatocytes, so that the participating P450 isoform(s) could be more easily identified.

Several analytical methods have been used to detect parathion and associated metabolites. High performance liquid chromatography (HPLC) techniques, either reverse or normal phase, with u.v. detection have been most often employed since all three compounds contain a benzene ring moiety and therefore absorb well in the ultra-violet range of the spectrum. A study by Butler and Murray (1983) measured paraoxon and *p*-

Figure 8.4



nitrophenol production following incubation with [^{14}C]parathion and separation by HPLC. This method could infer increased sensitivity compared to other techniques, although the costs involved in obtaining [^{14}C]parathion would be prohibitive for many researchers.

The analytical method chosen for this study was similar to that of Anderson *et al* (1992) in which microsomal incubations were terminated by protein precipitation and then analytes separated and quantitated by reverse phase HPLC with u.v. detection.

Reagents

50mM Tris buffer (pH 7.4), (chapter 7). Perchloric acid (20% v/v) was prepared by diluting the commercially available concentrated solution (60% v/v) with water. NADPH (6mg/ ml) was freshly prepared in buffer prior to each assay and kept on ice. Stock solutions of parathion (50mM and 5mM) and paraoxon (100 μM) were prepared in methanol from commercial (Greyhound Chromatography, Merseyside, U.K.) parathion (4.33M) and paraoxon (4.64M). Both are oily liquids. Stock *p*-nitrophenol (100 μM) was also prepared in methanol.

The influence of protein, time and substrate concentration

Parathion metabolism by rat liver microsomes was carried out at saturating substrate concentration (200 μM) in order to measure V_{max} rates of paraoxon and *p*-nitrophenol formation, and also at 20 μM . Under these conditions the rate of *p*-nitrophenol and paraoxon formation was linear with time and protein concentration to 10 minutes and 0.4mg protein per incubation, respectively (figures 8.5 and 8.6). Preliminary experiments showed that the volume of methanol (2 μl) required to

Figure 8.5

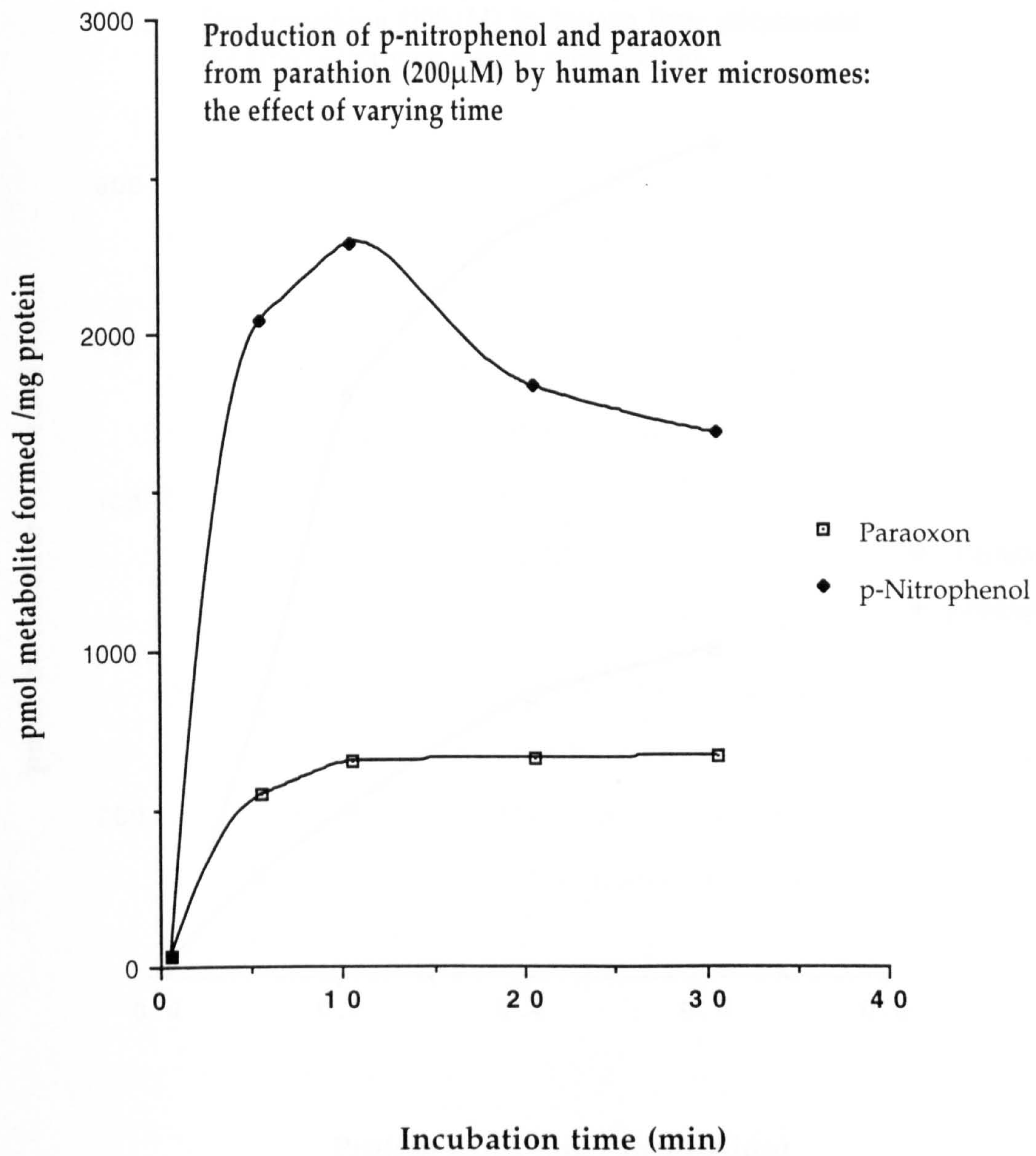
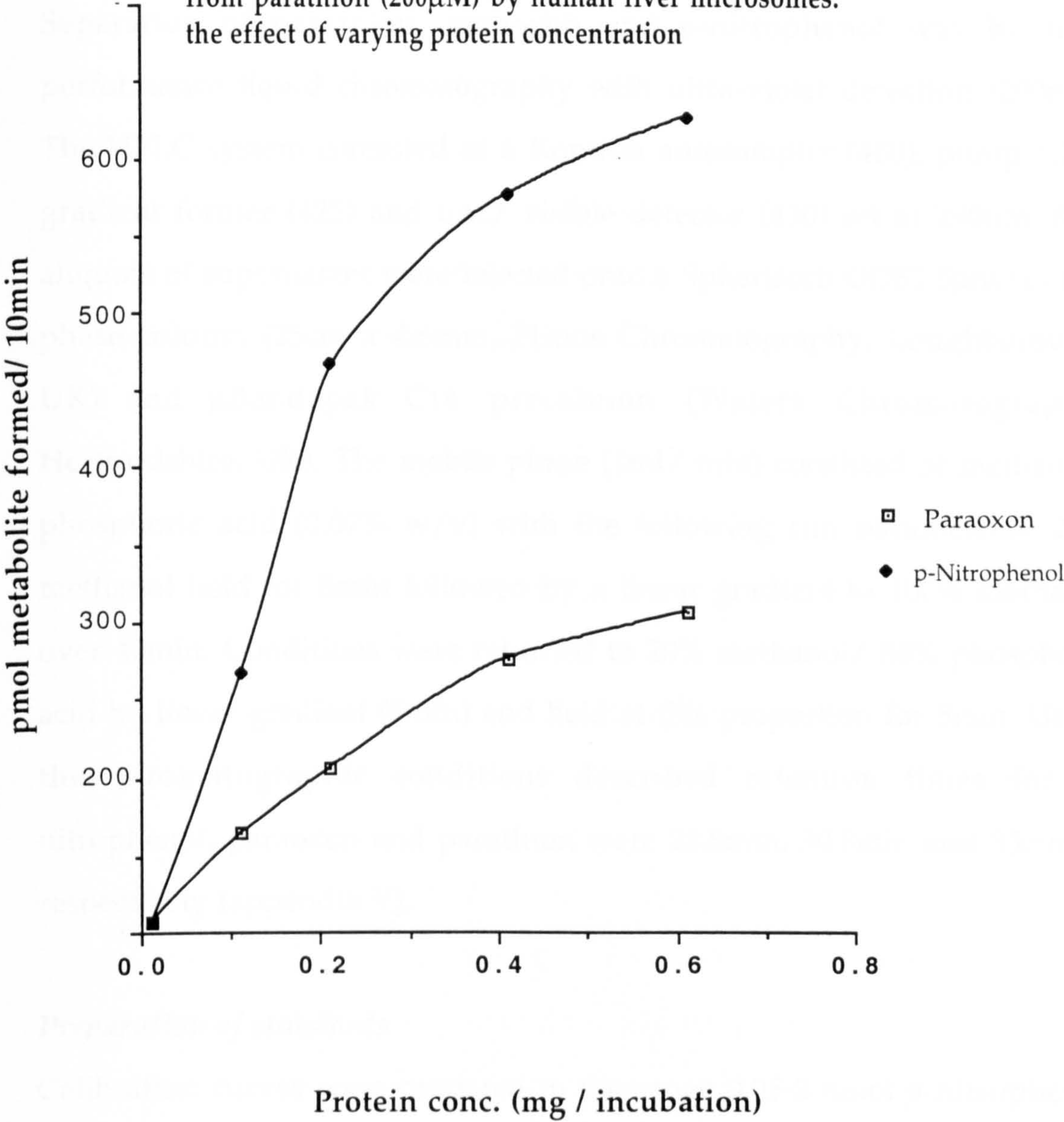


Figure 8.6

Production of p-nitrophenol and paraoxon from parathion (200μM) by human liver microsomes: the effect of varying protein concentration



introduce the substrate did not affect P450-mediated metabolism of parathion.

High Performance Liquid Chromatography Conditions

Separation of parathion, paraoxon and *p*-nitrophenol was by high performance liquid chromatography with ultra-violet detection (290nm). The HPLC system consisted of a Kontron autosampler (460), pump (420), gradient former (425) and u.v./ visible detector (430) set at 290nm. 80µl aliquots of supernatant were injected onto a Spherisorb ODS1 5µm reverse phase column (25cm x 4.6mm, Fisons Chromatography, Loughborough, UK) and µBondapak C18 precolumn (Waters Chromatography, Hertfordshire, UK). The mobile phase (1ml/ min) consisted of methanol/ phosphoric acid (0.07% w/v) with the following run parameters:- 20% methanol held for 5min followed by a linear gradient to 100% methanol over 40min. Conditions were returned to 20% methanol/ 80% phosphoric acid by linear gradient (5min) and held at this proportion for 5min. Using the chromatographic conditions described retention times for *p*-nitrophenol, paraoxon and parathion were 23.8min, 30.8min and 33.5min, respectively (appendix V).

Preparation of standards

Calibration curves were prepared in the range 0.05-2 nmol *p*-nitrophenol and paraoxon per incubation in the presence and absence of 0.4mg microsomal protein but without NADPH. Parathion was not included in these standards. Standards were then treated in the same manner as the samples, without incubation at 37°C. 80µl aliquots were injected onto the HPLC system. The analytical limit of detection was 10pmol/incubation and 35pmol/incubation for *p*-nitrophenol and paraoxon, respectively.

Incubation procedure

The incubation system consisted of liver microsomes in 0.5ml of Tris/calcium buffer (pH 7.4) containing 0.3mg NADPH. Control incubations were carried out as described above except that microsomal protein or NADPH was omitted. The reaction was started by addition of 2µl parathion stock solutions (5mM or 50mM) in methanol which gave final parathion concentrations of 20µM or 200µM, respectively. The incubations were conducted at 37°C in a shaking water bath and terminated after a suitable period by adding an equal volume of 20% (v/v) perchloric acid followed by rapid cooling on ice. Samples were centrifuged at 1 000g for 15min and then the clear supernatants carefully removed to clean vials prior to analysis by HPLC for *p*-nitrophenol and paraoxon formation.

8.4.1 Results and discussion

Linear calibration curves were produced for paraoxon (figure 8.7) and *p*-nitrophenol (figure 8.8) over the range studied (0.5-5 nmol/ incubation). There was no difference between the curves for paraoxon (or *p*-nitrophenol) measured in the presence and absence of microsomal protein. However, it is possible that the paraoxon concentration of the standards would have been reduced by binding to carboxylesterases if they had been incubated at 37°C. It is therefore possible that rates for paraoxon formation determined by this method may have been slightly underestimated. In this context, Norman *et al* demonstrated that up to 20% of the paraoxon formed *in vitro* could be bound to the microsomes and therefore accurate measurements of paraoxon formation should include the concentration of free metabolite in the incubation media plus the amount bound to the microsomes.

Figure 8.7

A typical paraoxon standard curve.

Data points are the mean of duplicate determinations.

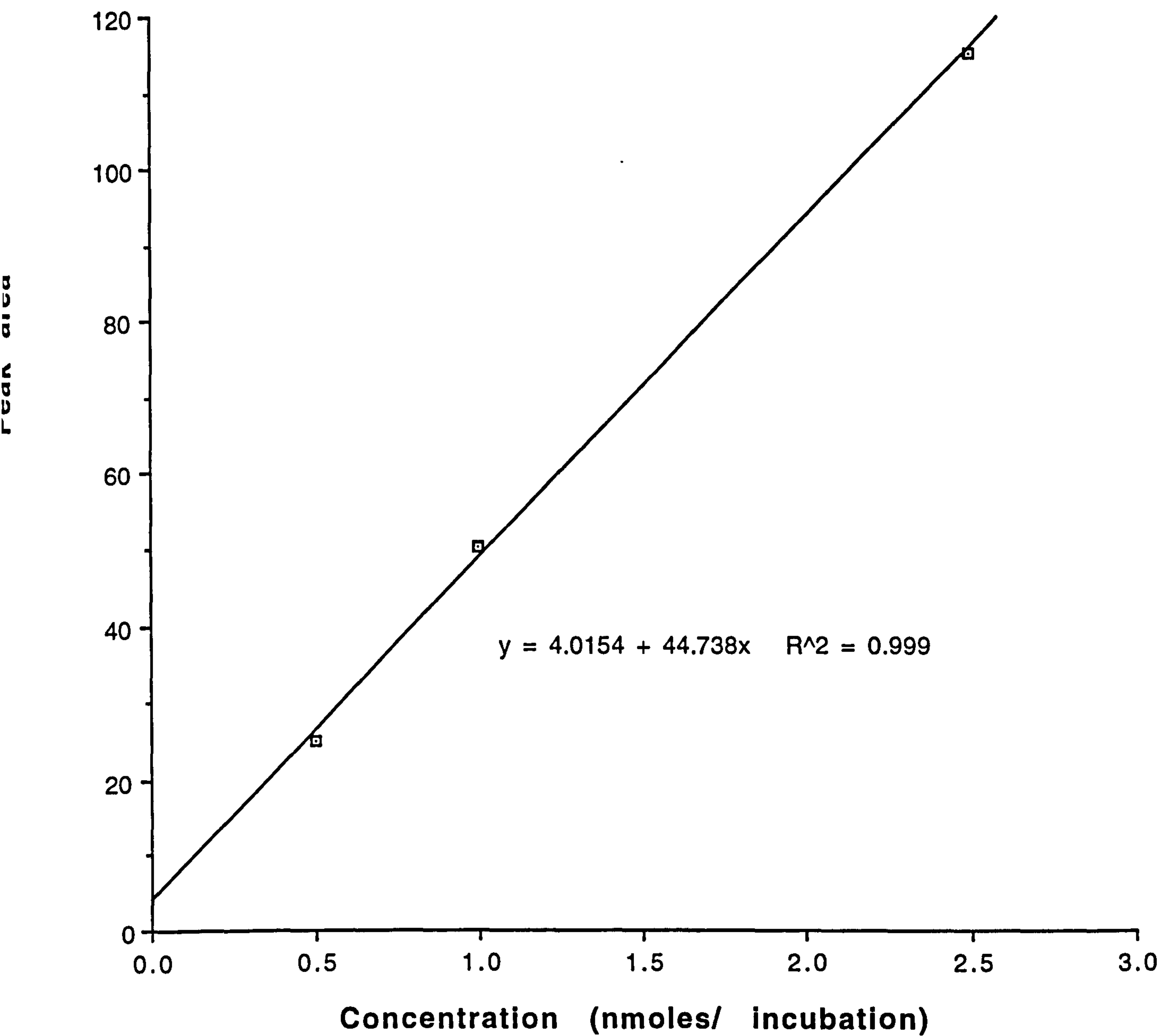
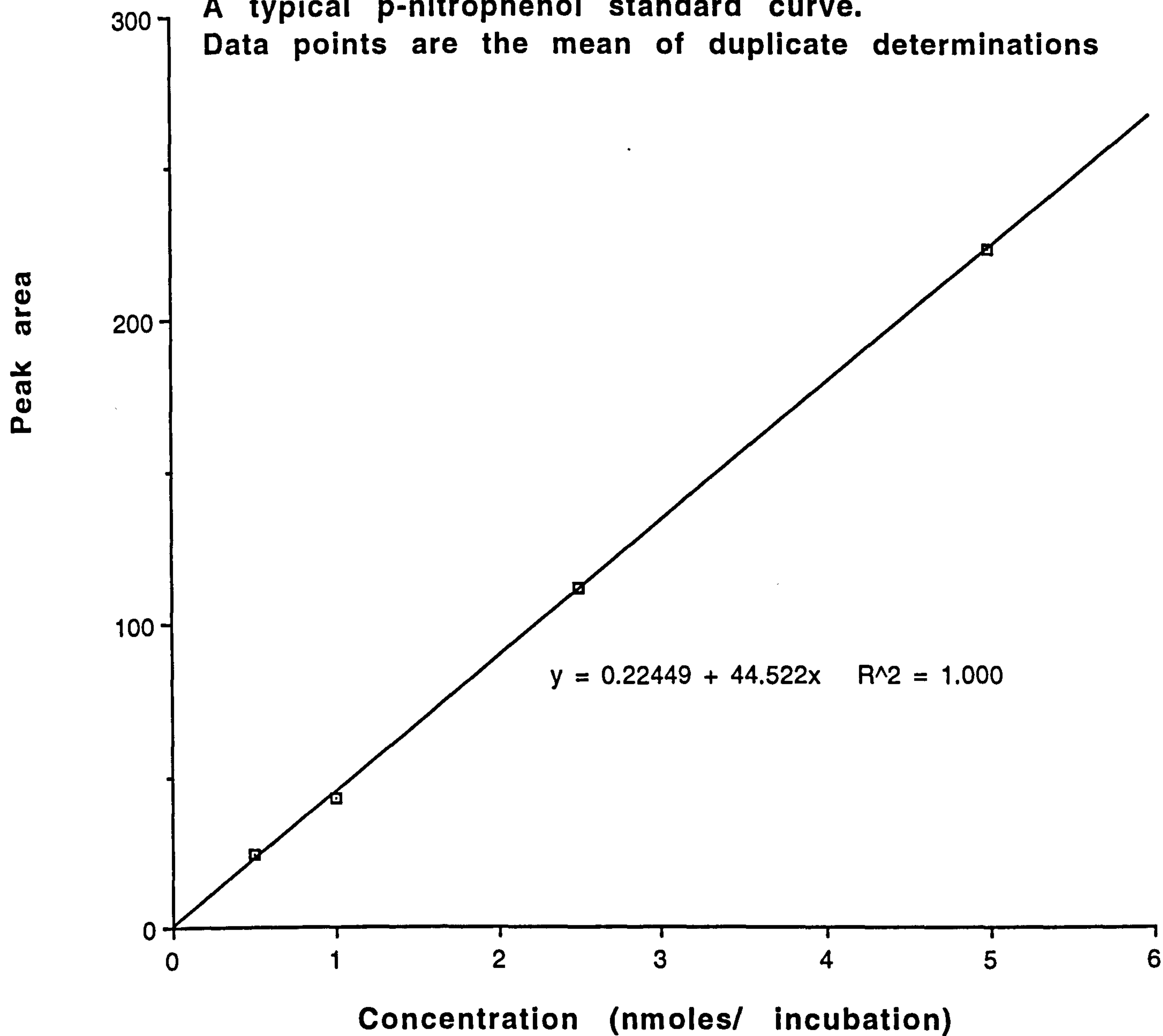


Figure 8.8

A typical p-nitrophenol standard curve.

Data points are the mean of duplicate determinations



Initial experiments showed that the rate of parathion metabolism to *p*-nitrophenol and paraoxon rapidly departed from linearity with respect to protein and time. This observation indicated rapid inhibition of the metabolism of parathion which corroborates the early hypothesis of Norman *et al* (1974). These researchers suggested that the sulphur atom of phosphorothioates is released during their oxidative metabolism and transferred to the haem moiety of P450 leading to amino acid modification and enzyme destruction.

8.5 Measurement of flavin monooxygenase activity

The *N*-oxidation of dimethylaniline (DMA) has been used as a sensitive marker reaction for flavin monooxygenase (FMO) activity (Sadeque *et al*, 1993). The flavin monooxygenases are located in the microsomal fraction of mammalian liver and their capacity to form dimethylaniline *N*-oxide was determined in this study by a modification of the gas chromatographic method of Damani and Houdi (1988).

Reagents

Glycine buffer, pH 8.5 (chapter 7). Stock solutions of *N,N*-dimethylaniline (0.5M) and *N*-ethylaniline (1mM, internal standard) were prepared in methanol prior to use. A solution of authentic *N,N*-dimethylaniline *N*-oxide (1mM in water) was provided by Dr A Rettie (Seattle, USA). NADPH (6mg/ ml) was freshly prepared in buffer prior to each assay and kept on ice.

The influence of protein, time and substrate concentration

The substrate concentration, 12.5mM *N,N*-dimethylaniline, used in this study was similar to that reported in the literature (Sherratt and Damani,

1989) although it was recognised that oxidation of a xenobiotic by FMO is a second order reaction not saturable by substrate. Under the conditions described below the rates of dimethylaniline *N*-oxide formation were linear with time and protein concentration to 10 minutes and 0.15mg/incubation, respectively.

Procedure

A Hewlett Packard (HP 5890A, series II) gas chromatograph (GC) equipped with an automatic injector (HP 7673), integrator (HP 3396A) and flame ionisation detector (FID) was employed. Injection (2µl, splitless) was achieved into a heated (230°C) injection port connected to a capillary HP-1 column (25m x 0.32mm, 0.52µm film thickness). The detector temperature was 200°C, the oven temperature commenced at 60°C and followed a linear gradient (1°C/min) until 90°C where it was held for 50 minutes. The carrier gas was nitrogen (1.3 ml/ min, total flow). Under these conditions the retention times obtained for N, N-dimethylaniline (dimethylaniline *N*-oxide) and N-ethylaniline (internal standard) were 17.8 and 21.1 minutes, respectively.

Control liver microsomes were equilibrated to 37°C in glass test tubes with NADPH (0.5mg) and glycine buffer to a final volume of 0.2ml. Analysis was carried out in triplicate. The reaction was started on addition of 2.5µmoles (5µl) of N, N-dimethylaniline, to give a final substrate concentration of 12.5mM. After a suitable period the reaction was terminated by addition of 0.1ml of sodium hydroxide (5M), the tubes were vortexed and placed on ice. The mixture was then twice extracted (vortex, 30 seconds) with diethyl ether (5ml) to remove excess N, N-dimethylaniline, the organic layer was removed to waste on each occasion. Following addition of a third aliquot (5ml) of diethyl ether, the tubes were

vortexed, centrifuged 10min x 1 000g, the organic layer removed to waste and 0.15ml N-ethylaniline (1mM, internal standard) added. 1.0ml of HCl (1.2M) and 20µl Titanium (III) chloride (15%, w/v) was then added and the mixture allowed to stand for 5 minutes, this reduced dimethylaniline N-oxide, produced by the microsomes, back to N, N-dimethylaniline.

Finally, 1ml of sodium hydroxide (5M) was added, the mixture vortexed and then centrifuged for 10 minutes x 1 000g to remove any remaining protein. The resulting supernatant was removed to clean tubes containing approximately 1g sodium chloride and 14ml hexane and vortexed for one minute. The hexane (top) layer was then transferred to clean tubes and concentrated to approximately 250µl under nitrogen. This was removed to clean G.C. vials for detection of N, N-dimethylaniline (dimethylaniline N-oxide) and N-ethylaniline (internal standard).

Quantitation of the reaction

A standard curve was constructed for authentic dimethylaniline N-oxide (20 - 80 nmol/ incubation) in the presence and absence of boiled microsomes (0.1mg protein) and treated in the same manner as the samples from the final addition of 1.0ml sodium hydroxide (5M). Inspection of the standard curves for dimethylaniline N-oxide in the presence and absence of microsomal protein showed that they were similar. Standards were expressed as area dimethylaniline N-oxide/ area internal standard ratios. The amount of dimethylaniline N-oxide produced by the microsomal samples was quantitated by comparison to the standard curve. Enzyme activity was expressed as nmol dimethylaniline N-oxide produced/ min/ mg microsomal protein.

Paraoxon is the most common substrate for studying paraoxonase (aryldialkylphosphatase) activity and measurement of its hydrolysis to *p*-nitrophenol is considered to be a marker reaction for the detoxification of organophosphates. Paraoxonase has been located exclusively in the microsomal fraction of mammalian liver (McCracken *et al*, 1990) and its activity was determined in this study by a modification of the method of Eckerson (1983) in which appearance of *p*-nitrophenol was monitored photometrically.

Reagents

Glycine/calcium buffers (chapter 7). Paraoxon was purchased from Greyhound Chromatography (Merseyside, U.K.) as an oily liquid (4.6M) and a working stock (100mM) prepared by diluting 1:45 in dry acetone prior to use.

The influence of pH

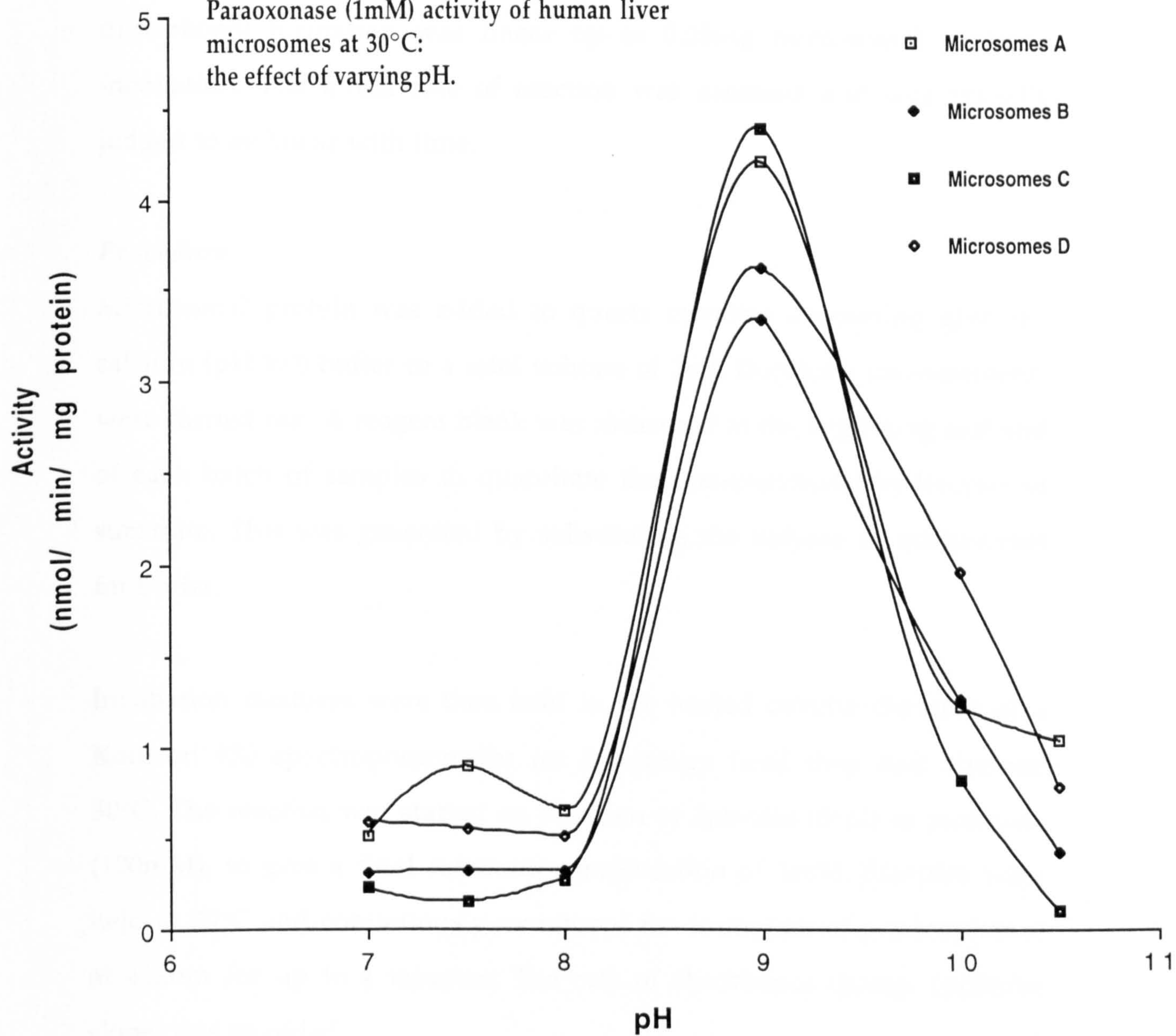
Initial studies with human liver microsomes (n = 4) determined the optimal pH for the enzyme.

Paraoxonase activity was assessed at pHs 7.0, 7.4, 8.0, 9.0, 10.0 and 10.5. Approximately 0.2mg protein was incubated with paraoxon (1mM) at 30°C and formation of *p*-nitrophenol monitored continuously, as described below.

Activity was shown to be optimal at pH 9.0 with rates at pH 7.4 approaching the limit of sensitivity for the assay (figure 8.9).

Figure 8.9

Paraoxonase (1mM) activity of human liver microsomes at 30°C:
the effect of varying pH.



The influence of protein, time and substrate concentration

The paraoxon concentration (1mM) used in this study was more than two-fold greater than the reported K_m (0.4mM, Gil *et al*, 1993) to ensure that V_{max} rates were measured. Under these conditions at pH 9.0 the rate of *p*-nitrophenol formation was linear up to 0.25mg microsomal protein/incubation. The initial rate of reaction was assessed and was visually judged to be linear with time.

Procedure

Microsomal protein was added to quartz cuvettes containing glycine/calcium (pH 9.0) buffer to a total volume of 3ml. Duplicate measurements were carried out. A reagent blank was measured at the beginning and end of each batch of samples to quantitate the non-enzymatic hydrolysis of substrate. This was generated by substituting the volume of microsomes for buffer.

Incubation mixtures were then held in the heated cuvette chamber of a Kontron 930 spectrophotometer for 5 minutes until they had attained 30°C. The reaction was started on addition of 3μmoles (30μl) of paraoxon (100mM), to give a final substrate concentration of 1mM. Samples were held at 30°C and continuously monitored for formation of *p*-nitrophenol at 412nm for up to 4 minutes. The rate of absorbance change (positive slope) was recorded.

Quantitation of the reaction

Enzyme activity was determined by an adaptation of Beer-Lamberts law. Under the assay conditions described:

E = 0.128 absorbance units (AU) for a 10nmol/ ml solution *p*-nitrophenol in phosphate buffer (pH 7.4), i.e. 1.0 AU = 78.1nmol *p*-nitrophenol/ ml.

E = 0.176 absorbance units (AU) for a 10nmol/ ml solution *p*-nitrophenol in glycine buffer (pH 9.0), i.e. 1.0 AU = 56.8nmol *p*-nitrophenol/ ml.

A = the rate of reaction (Δ AU/ min, slope)

C = mg microsomal protein/ 1ml incubation mixture

L = cuvette pathlength (1cm)

Therefore,

nmol *p*-nitrophenol formed/ min/ mg protein =

$$\frac{\Delta \text{AU/ min} \times 78.1}{\text{mg protein/ 1ml incubation}} \quad \text{at pH 7.4}$$

$$\frac{\Delta \text{AU/ min} \times 56.8}{\text{mg protein/ 1ml incubation}} \quad \text{at pH 9.0}$$

8.7 Measurement of phenylvalerate hydrolase activity

The hydrolytic activity of serine-containing esterases (carboxylesterases and cholinesterases) of liver microsomes was measured in this study by a sensitive spectrophotometric method based on that of Johnson (1977) modified by Mutch *et al*, 1995. In this method phenol liberated on hydrolysis of the substrate, phenylvalerate, was coupled to 4-aminoantipyrine and the colour developed by addition of potassium ferricyanide. The resultant red aminoantipyrine dye was measured photometrically.

Reagents

Tris/ EDTA (pH 8.0) and 500mM Tris (pH 8.5) buffers (chapter 7). Phenylvalerate was synthesized by Mutch (1992) following the method of Johnson (1977) and a stock solution (15mg/ ml) prepared in redistilled dimethylformamide. Phenylvalerate stock solution was diluted 1:30 in TritonX 100 (0.03% (v/v) in water) prior to use. 4-aminoantipyrine (0.5% (v/v) in 500mM Tris buffer) was diluted 1:20 in stock sodium dodecylsulphate (1.05% (w/v) in Tris/EDTA buffer) prior to use. A stock solution of potassium ferricyanide (4% (v/v) in water) was diluted 1:10 in water for use.

The influence of protein, time and substrate concentration

Liver microsomal phenylvalerate hydrolase activity was carried out at saturating substrate concentration (1.4mM) to ensure that V_{max} rates were measured. Under these conditions the rate of phenol formation was linear with time and protein concentration to 15 minutes and 100ng/ incubation, respectively.

Procedure

Liver microsomes were equilibrated to 37°C in 0.2ml Tris/EDTA buffer in parallel with a reagent blank which was carried out in order to determine non-enzymatic production of phenol. This was generated by substituting liver microsomal suspension with an equal volume of Tris/ EDTA buffer and following through the assay. The reaction was started on addition of 0.2ml phenylvalerate in TritonX 100, to give a final substrate concentration of 1.4mM. After a suitable period the reaction was stopped by addition of 0.2ml 4-aminoantipyrine/ sodium dodecylsulphate working stock and the tubes placed on ice. Phenol liberated during the incubation period was coupled to 4-aminoantipyrine and the red colour developed on

addition of 0.1ml potassium ferricyanide working solution. The absorbance at 510nm was read in quartz micro-cuvettes against a blank (reagents other than phenylvalerate) in the reference position. A Kontron 930 double-beamed spectrophotometer was employed.

Quantitation of the reaction

A standard curve was constructed for authentic phenol (20 - 50 nmol/incubation) in the absence of microsomal protein and treated in the same manner as the samples. The amount of phenol produced by the microsomal incubations was quantitated by comparison to the standard curve. Enzyme activity was expressed as mmol phenol produced/ min/ mg microsomal protein.

8.8 Measurement of glutathione-S-transferase activity

A convenient spectrophotometric method (Habig *et al*, 1974) has been developed for the analysis of glutathione-S-transferase based on the enzyme catalysed condensation of glutathione with the model substrate 2,4-dinitro-1-chlorobenzene. The product formed (2,4-dinitrophenyl-glutathione) absorbs at 340nm. 2,4-dinitro-1-chlorobenzene is a good substrate for most of the glutathione-S-transferase isoforms and therefore the observed activity will be a composite result of the isoforms present in the tissue preparation.

Reagents

100mM potassium phosphate buffer, pH 6.5, (chapter 7). Stock solutions of reduced glutathione (GSH, 30mM in buffer) and 2,4-dinitro-1-chlorobenzene (DNCB, 30mM in ethanol) were prepared prior to each assay and stored on ice.

The influence of protein, time and substrate concentration

An apparent K_m of $250\mu\text{M}$ was determined for formation of the DNCB adduct by human liver cytosol ($n=1$) using an Eadie Hofstee plot based upon data from a range of DNCB concentrations ($0.075\text{--}2.0\text{mM}$) in which conditions of linearity with respect to time and protein concentration were employed. The present study used a saturating substrate concentration of 1mM to ensure V_{max} rates. Under these conditions the rate of DNCB adduct formation was linear with protein to $75\mu\text{g/ incubation}$; the initial rate of reaction was assessed which was visually judged to be linear with time.

Procedure

Liver cytosol was equilibrated to 37°C with $33\mu\text{l}$ GSH (30mM), $33\mu\text{l}$ DNCB (30mM) and phosphate buffer ($\text{pH } 6.5$) to a total volume of 1.0ml . This gave a final substrate concentration of 1mM . The reaction mixture was prepared in duplicate in disposable microcuvettes. A reagent blank was carried out with every run and generated by replacing cytosolic protein with the same volume of buffer and placing the cuvette in the reference position of the spectrophotometer. This automatically subtracted non-enzymatic formation of the DNCB adduct ($<10\%$ of enzymatic formation) from enzymatic catalysis. The reaction was started on addition of cytosolic protein and the increase in absorbance at 340nm followed for about 3 minutes at 37°C .

Quantitation of the reaction

Using Beer's law (and a cuvette pathlength of 1cm), the specific activity of DNCB glutathione-S-transferase is given by:

$P =$ protein concentration (mg) in 1ml incubation

E = 9.6mM (extinction coefficient for glutathione/ DNCB adduct at 340nm)

A = the rate of reaction (Δ absorbance units/ min, slope)

Therefore,

$\mu\text{mol product formed/ min/ mg protein} =$

$$\frac{A}{9.6 \times P}$$

SECTION III:

EXPERIMENTAL STUDIES

Chapter 9

Chapter 9 Rat induction studies *in vivo*

9.1 Introduction

The inclusion of an animal component in xenobiotic metabolism studies has several advantages. Particularly, rat enzyme systems can be modulated *in vivo* and *in vitro* in order to elucidate the metabolic pathways involved in a particular reaction which is not possible for human studies. It was therefore decided to study the metabolising capacity of rat liver in order to complement the data derived from the limited supply of human tissue. The methods for measurement of these enzymes had been previously established to ensure valid analysis.

Initial studies were concerned with the characterisation of control rat liver microsomes for the cytochromes P450, FMO and esterases which may be involved in parathion metabolism. Next the capacity of control microsomes to metabolise parathion to paraoxon and *p*-nitrophenol at saturating substrate concentration using optimal assay conditions was defined.

The efficacy of the induction regimen was assessed by measuring the increase in activity of the cytochromes P450 and esterases compared to control. Increased parathion metabolism by the various groups of induced liver microsomes was then determined in order to investigate the P450 isoform(s) involved in the catalysis of this phosphorothioate.

As the literature (Guengerich, 1977) suggested the involvement of phenobarbital-inducible isoforms (CYPs 2B and 3A) one group of rats was to be induced by phenobarbital. Another was induced by dexamethasone

in order to increase the level of the CYP3A subfamily (Okey *et al*, 1990), the predominant human liver P450 isoform. Cytochromes P450 1A and 2E1 were induced with β -naphthoflavone (a 3-methylcholanthrene-type inducer) and ethanol pre-treatment, respectively.

9.2 Methods

9.2.1 Induction regimen

Male Wistar rats weighing approximately 250g were randomised into 6 treatment groups: saline control, phenobarbital, ethanol, corn oil control, dexamethasone and β -naphthoflavone. Within each group of five each rat was tail-marked and weighed. The saline-treated and corn oil-treated animals served as controls for the phenobarbital and ethanol groups, and dexamethasone and β -naphthoflavone-treated groups, respectively. Dosing regimens were adapted from Verschoyle *et al* (1993) and Fentem *et al* (1991). All animals were sacrificed by cervical dislocation 24 hours after the last dose, their livers weighed and then stored at -70°C.

Saline control group received 2ml normal saline/ kg body weight/ day by i.p. injection for 3 days.

Phenobarbital group received 80mg sodium pentobarbital in normal saline/ kg body weight/ day by i.p. injection for 3 days.

Ethanol group received 15% ethanol (v/v) in their drinking water for 5 days.

Corn oil control group received 2ml corn oil/ kg body weight/ day by oral gavage for 3 days.

Dexamethasone group received 300mg dexamethasone in corn oil/ kg body weight/ day by oral gavage for 4 days.

β-Naphthoflavone group received 80mg β-naphthoflavone/ kg body weight/ day by oral gavage for 3 days.

9.2.2 Biochemical methods

Ethoxyresorufin- and pentoxyresorufin-O-dealkylase activity

Approximately 0.05mg or 0.15mg microsomal protein was incubated with ethoxyresorufin or pentoxyresorufin, respectively, in 1ml phosphate buffer (pH 7.4). Formation of the common metabolite, resorufin, was monitored continuously at 37°C for about 5min.

p-Nitrophenol hydroxylase activity

Approximately 0.25mg - 0.5mg protein in 0.5ml potassium phosphate buffer (pH 6.8) was incubated with *p*-nitrophenol for 10min at 37°C. Formation of 4-nitrocatechol was determined by u.v. detection (250nm) following separation by reverse-phase HPLC.

Nifedipine oxidase activity

Approximately 0.5mg microsomal protein in 1ml KCl/ phosphate buffer (pH 7.4) was incubated with nifedipine for 10min. The reaction was terminated by addition of 400μl Tris buffer (pH 9.0) and samples analysed for formation of the metabolite M1 by GC with electron capture detection.

Flavin monooxygenase activity

Approximately 0.1mg microsomal protein was incubated with N, N-dimethylaniline (12.5mM) in a total volume of 0.2ml glycine buffer, pH 8.5. The reaction was terminated after 10min and formation of dimethylaniline N-oxide determined by GC with FID detection.

Paraoxonase activity

Approximately 0.1mg microsomal protein was incubated with paraoxon in a total volume of 3ml glycine buffer (pH 9.0) held at 30°C. Production of the metabolite, *p*-nitrophenol, was monitored continuously at 412nm for about 3min and the rate of reaction determined.

Phenylvalerate hydrolase activity

Liver microsomes were diluted appropriately so that approximately 0.5ng microsomal protein was incubated with phenylvalerate in a total volume of 0.4ml Tris/ EDTA buffer, pH 8.0. Incubations were terminated after 10min and formation of phenol determined photometrically at 510nm.

Parathion metabolism

Approximately 0.3mg microsomal protein was incubated for 10min in the presence of NADPH and parathion (20μM and 200μM, final concentration) with Tris/ calcium buffer (pH 7.4) to 0.5ml. Samples containing either no microsomal protein or no NADPH were carried through the procedure in parallel. Formation of *p*-nitrophenol and paraoxon was determined by HPLC with u.v. detection.

Data from each of the treatment groups were compared to appropriate control measurements and percentage change in enzyme levels calculated. Methods for liver microsome preparation, and total P450 and enzyme measurements are described in chapters 7 and 8, respectively. The microsomal protein concentration of each of the preparations was determined by the method of Lowry adapted by Peterson (1977) using bovine serum albumin as standard, as described in chapter 7.

9.3 Results

9.3.1 Control enzyme activities

The control measurements for P450, FMO and esterase activities given in this chapter relate to the saline treatment group of rats of the induction study. Control P450 content was 0.534 ± 0.062 nmol/ mg protein.

Table 9.1 Ethoxy- and pentoxyresorufin-O-dealkylase activities of control rat liver microsomal fractions. Results are the mean \pm sem, n=5. The substrate concentrations were 2 μ M.

Expression of activity	Ethoxyresorufin-O-dealkylase activity	Pentoxyresorufin-O-dealkylase activity
pmol/ min/ mg protein	28.1 ± 2.4	4.2 ± 0.33
pmol/ min/ g liver	317.8 ± 33.5	47.0 ± 4.9
pmol/ min/ nmol total P450	53.8 ± 4.1	8.0 ± 0.66

The ethoxyresorufin- and pentoxyresorufin-O-dealkylase activities for control rat liver microsomes, measured at saturating substrate concentrations, are given in table 9.1. Activities quoted in the literature for ethoxyresorufin-O-dealkylase activity range from 12pmol/ min/ mg protein (Walther *et al*, 1987) to about 60pmol/ min/ mg protein (Toftgard and Nilsen, 1982; Gulyaeva *et al*, 1993) and the values determined in this study (28.1 ± 2.4 pmol/ min/ mg protein) are within this range. Pentoxyresorufin-O-dealkylase activity, at 4.2pmol/ min/ mg protein, was about seven fold lower than that obtained for the O-dealkylation of ethoxyresorufin, whether activity was expressed /mg protein, /g liver or /nmol total P450.

Table 9.2 *p*-Nitrophenol hydroxylase activities of control rat liver microsomal fractions. Results are the mean \pm sem, n=5. The substrate concentration was 200 μ M.

Expression of activity	<i>p</i> -Nitrophenol hydroxylase activity
nmol/ min/ mg protein	0.707 \pm 0.065
nmol/ min/ g liver	7.79 \pm 0.66
nmol/ min/ nmol total P450	1.36 \pm 0.13

Control rat liver microsomes produced 0.707 \pm 0.065 nmol 4-nitrocatechol/ min/ mg microsomal protein at saturating substrate concentration (table 9.2) which compares with the rate of 0.740 \pm 0.07 nmol 4-nitrocatechol/ min/ mg microsomal protein reported by Reinke and Moyer (1985) for Sprague Dawley rats.

Table 9.3 Nifedipine oxidase activities of control rat liver microsomal fractions. Results are the mean \pm sem, n=5. The substrate concentration was 100 μ M.

Expression of activity	Nifedipine oxidase activity
nmol/ min/ mg protein	1.74 \pm 0.12
nmol/ min/ g liver	23.7 \pm 2.6
nmol/ min/ nmol total P450	3.41 \pm 0.41

Non-enzymatic formation of M1 from nifedipine was <5% of that produced by liver microsomes using the conditions described above. This value was subtracted from that produced by the microsomal incubations. Literature quotes for the rate of nifedipine oxidation by mature male rat liver microsomes vary widely. For example, Niwa *et al* (1995) gave a value of 2.69 nmol/ min/ mg protein, while Yamazaki *et al* (1996) reported 3.40 nmol/ min/ mg protein. A study by Guengerich *et al* (1986) determined a rate of 5.34 nmol/ min/ mg protein for adult male rats and 1.65 nmol/

min/ mg protein in immature (100g) animals. In the present study control rat liver microsomes produced 1.74 ± 0.12 nmol/ min/ mg microsomal protein at saturating substrate concentration (table 9.3) which compares to 0.82 ± 0.19 nmol/ min/ mg microsomal protein given in a recent study by Shimada *et al* (1997). The Shimada (1997) study also expressed the formation of M1 per nmol P450 and at 1.22 ± 0.28 , relates to that determined in this study (3.41 ± 0.41 nmol/ min/ nmol total P450).

Control liver FMO activity was 25.3 ± 1.39 nmol dimethylaniline N-oxide formed/ min/ mg protein (n=5) which is approximately 25 fold greater than that reported by Damani and Houdi (1988) for Sprague Dawley rats. This inconsistency may reflect differences in husbandry since dietary components, notably organic nitrogen and sulphur soft nucleophiles which are abundant in food derived from plants, are known to modulate FMO activity (Ziegler, 1993).

Table 9.4 Paraoxonase activity of control rat liver microsomal fractions measured at pH 7.4 and pH 9.0. Results are the mean \pm sem, n=5. Substrate concentration was 1mM.

Assay pH	Paraoxonase activity	
	(nmol/ min/ mg protein)	(nmol/ min/ g liver)
7.4	2.06 ± 0.38	26.2 ± 4.6
9.0	46.3 ± 4.9	591.9 ± 48.6

Non-enzymatic formation of *p*-nitrophenol from paraoxon was negligible under the conditions described above (pH 7.4 and pH 9.0). Preliminary experiments showed that pH 9.0 was optimal for measurement of rodent liver paraoxonase activity (see chapter 8). Enzyme activity was approximately twentytwo-fold higher at optimal pH compared to that at physiological conditions, pH 7.4. Plasma paraoxonase activity (64 ± 3

nmol/ min/ ml) for Wistar rats measured at optimal pH conditions (pH 10.5) was almost ten-fold lower than the liver activity at pH 9.0 (Mutch, 1992).

Table 9.5 Phenylvalerate hydrolase activities of control rat liver microsomal fractions. Results are the mean \pm sem, n=5. Substrate concentration was 1.4mM.

Expression of activity	Phenylvalerate hydrolase activity
mmol/ min/ mg protein	5.65 \pm 0.30
mmol/ min/ g liver	68.9 \pm 4.6

Non-enzymatic formation of phenol from phenylvalerate was <10% using the conditions described above. Control liver microsomal activities are given in table 9.5.

Table 9.6 Parathion metabolism by control rat liver microsomal fractions. Results are the mean \pm sem (n=5).

Parathion conc. (μ M)	Paraoxon formation (pmol/ min/ mg protein)	<i>p</i> -Nitrophenol formation (pmol/ min/ mg protein)
20	241 \pm 17	235 \pm 15
200	256 \pm 18	220 \pm 23

Formation of *p*-nitrophenol and paraoxon from parathion (20 μ M and 200 μ M) by control rat liver microsomes are shown in table 9.6. There was no difference between *p*-nitrophenol and paraoxon levels at either parathion concentration. The reaction was entirely dependent on microsomal protein and NADPH.

9.3.2 The effect of induction on physiological parameters

The effects of the inducers on physiological parameters are given in tables 9.7 and 9.8.

Table 9.7 Body weights of rats at the beginning and end of the induction study. Results are expressed as the mean \pm sem (n = 5).

Treatment group	Body weight at start (g)	Body weight at end (g)
Saline control	332 \pm 9	337 \pm 8
Phenobarbital	325 \pm 5	312* \pm 7
Ethanol	318 \pm 4	320 \pm 2
Corn oil control	356 \pm 10	363 \pm 11
Dexamethasone	331 \pm 14	278** \pm 13
β -Naphthoflavone	359 \pm 12	358 \pm 9

* significantly lower than weight at the start of the study and saline control, $p < 0.05$ (ANOVA with modified t-test)

** significantly lower than weight at the start of the study, β -naphthoflavone and corn oil control, $p < 0.05$ (ANOVA with modified t-test)

Table 9.8 Liver weight, microsomal protein recovery and total P450 content of the microsomes at the end of the induction study. Results are expressed as the mean \pm sem (n = 5).

Treatment group	Weight of liver (% body weight)	Weight of liver (g)	Microsomal recovery (mg protein/ g liver)	Total P450 content (nmol/ mg protein)
Saline control	5.04** \pm 0.14	16.7 \pm 0.46	12.2 \pm 0.73	0.534 \pm 0.06
Phenobarbital	5.18 \pm 0.18	16.1 \pm 0.57	22.0@ \pm 2.37	0.754@ \pm 0.07
Ethanol	4.37a \pm 0.07	14.0a \pm 0.21	19.7@ \pm 1.45	0.484 \pm 0.03
Corn oil control	4.59 \pm 0.14	16.7 \pm 0.52	15.3** \pm 0.55	0.818** \pm 0.03
Dexamethasone	6.00* \pm 0.41	16.7 \pm 1.15	16.4* \pm 0.59	1.030* \pm 0.10
β -Naphthoflavone	5.09* \pm 0.14	18.2* \pm 0.49	13.5 \pm 1.39	0.911 \pm 0.05

Comparisons were by ANOVA with modified t-test.

Weight of liver (% body weight)

**saline control significantly higher than corn oil control, p<0.05

a significantly lower than saline control, p<0.05

* significantly higher than corn oil control, p<0.05

Weight of liver

a significantly lower than saline control, p<0.05

* significantly higher than corn oil control, p<0.05

Microsomal protein recovery

**corn oil control significantly higher than saline control, p<0.05

@ significantly higher than saline control, p<0.05

* significantly higher than corn oil control, p<0.05

Total P450 content of microsomes

**corn oil control significantly higher than saline control, p<0.05

@ significantly higher than saline control, p<0.05

* significantly higher than corn oil control, p<0.05

At the end of the study it was noted that the dexamethasone-treated animals, and to some extent the phenobarbital group, were eating poorly and demonstrated general malaise. This was corroborated *post mortem* since both these groups showed significantly lower body weights in comparison to the start of the study (table 9.8). It was observed that the animals in the ethanol group drank normally and showed no significant change in body weight.

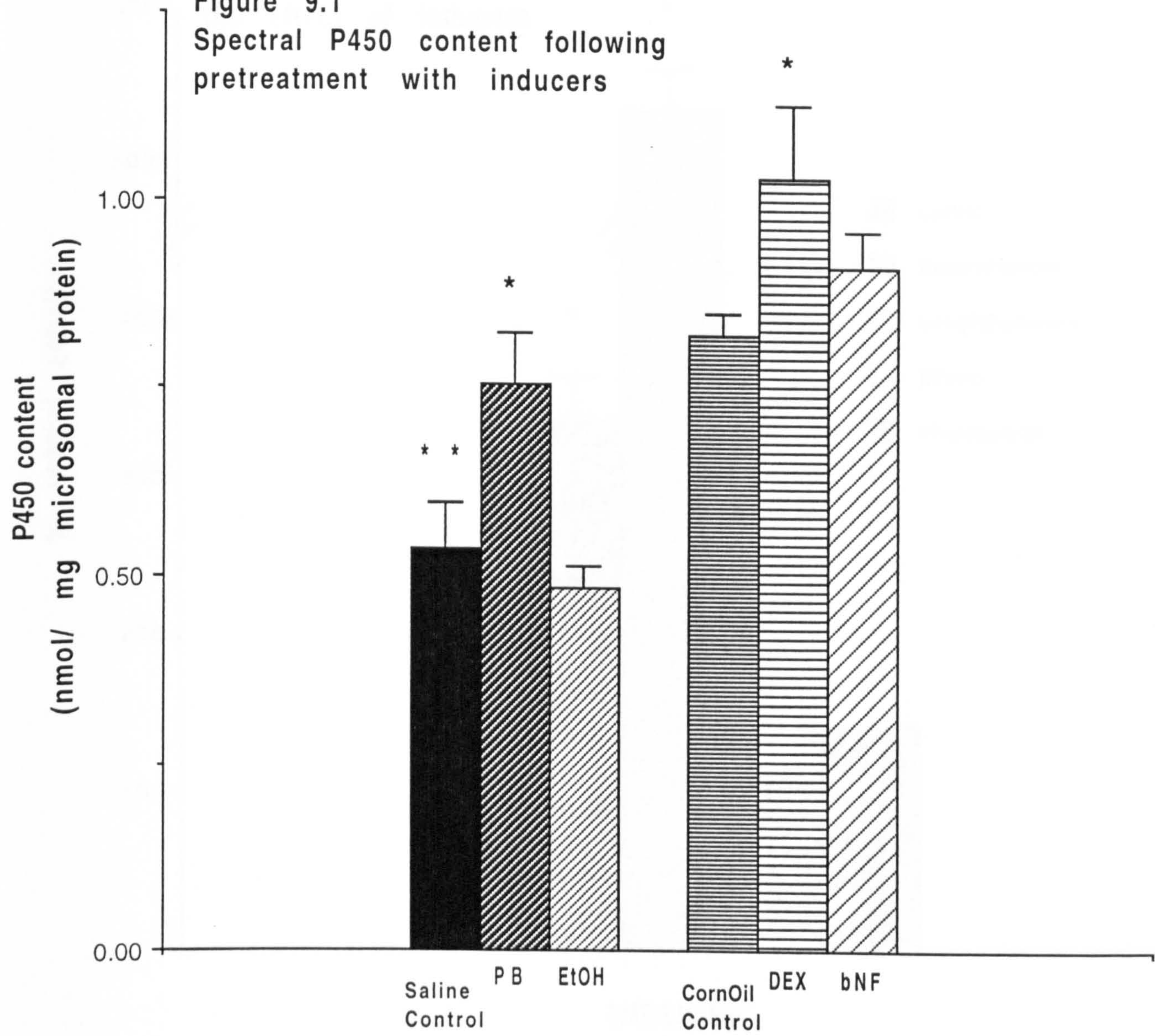
The corn oil control animals showed a significant loss in liver weight (as a percent of body weight), a higher microsomal protein recovery and a higher total P450 content compared to saline controls.

9.3.3 The effect of induction on the cytochromes P450 and esterases

The effect of inducers on total cytochrome P450 content is given in figure 9.1. As expected, ethoxy- and pentoxyresorufin-O-dealkylase activities were enhanced (approximately 50-fold) by β -naphthoflavone and phenobarbital pre-treatment, respectively, compared to their respective controls (figures 9.2 and 9.3). Also as expected *p*-nitrophenol hydroxylase (figure 9.4) and nifedipine oxidase (figure 9.5) activities were increased (approximately 2-fold and 5-fold) by ethanol and dexamethasone pre-treatments, respectively.

Unexpectedly, the corn oil control group showed lower pentoxyresorufin-O-dealkylase, *p*-nitrophenol hydroxylase and nifedipine oxidase activities compared to saline controls. Conversely, the ethoxyresorufin-O-dealkylase activity of corn oil controls was not significantly different from the saline control group. These data are given in table 9.9.

Figure 9.1
Spectral P450 content following
pretreatment with inducers

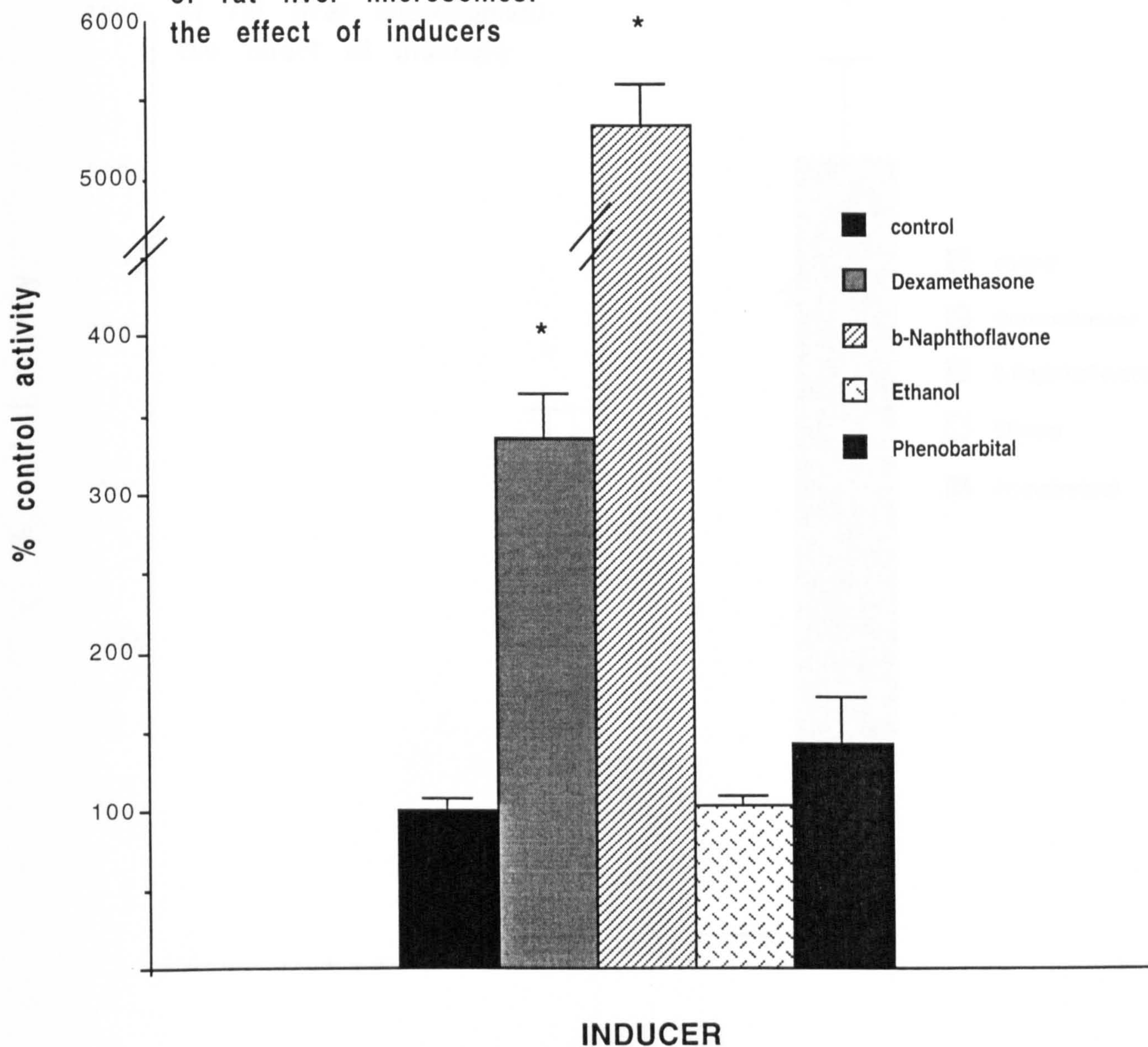


** $p < 0.05$, lower compared to corn oil control

* $p < 0.05$ compared to appropriate control

(ANOVA with modified t test)

Figure 9.2
Ethoxyresorufin-O-dealkylase activity
of rat liver microsomes:
the effect of inducers



* $p < 0.05$ compared to appropriate control

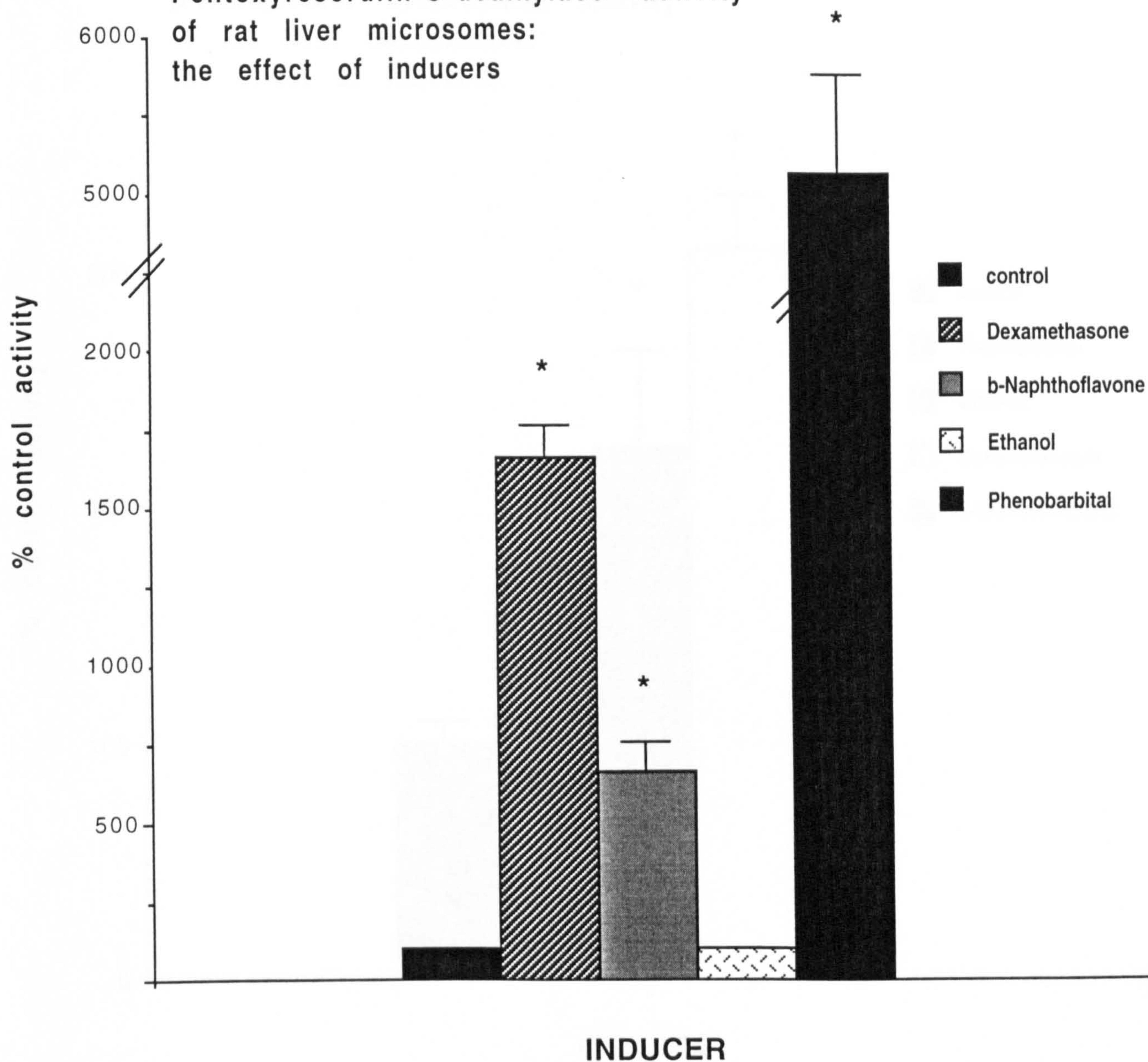
(ANOVA with modified t test)

Control (100%) activities

28.1 ± 2.4 pmoles/ min/ mg protein (Saline controls)

27.8 ± 3.4 pmoles/ min/ mg protein (Corn oil controls)

Figure 9.3
Pentoxeresorufin-O-dealkylase activity
of rat liver microsomes:
the effect of inducers



* $p < 0.05$ compared to appropriate control

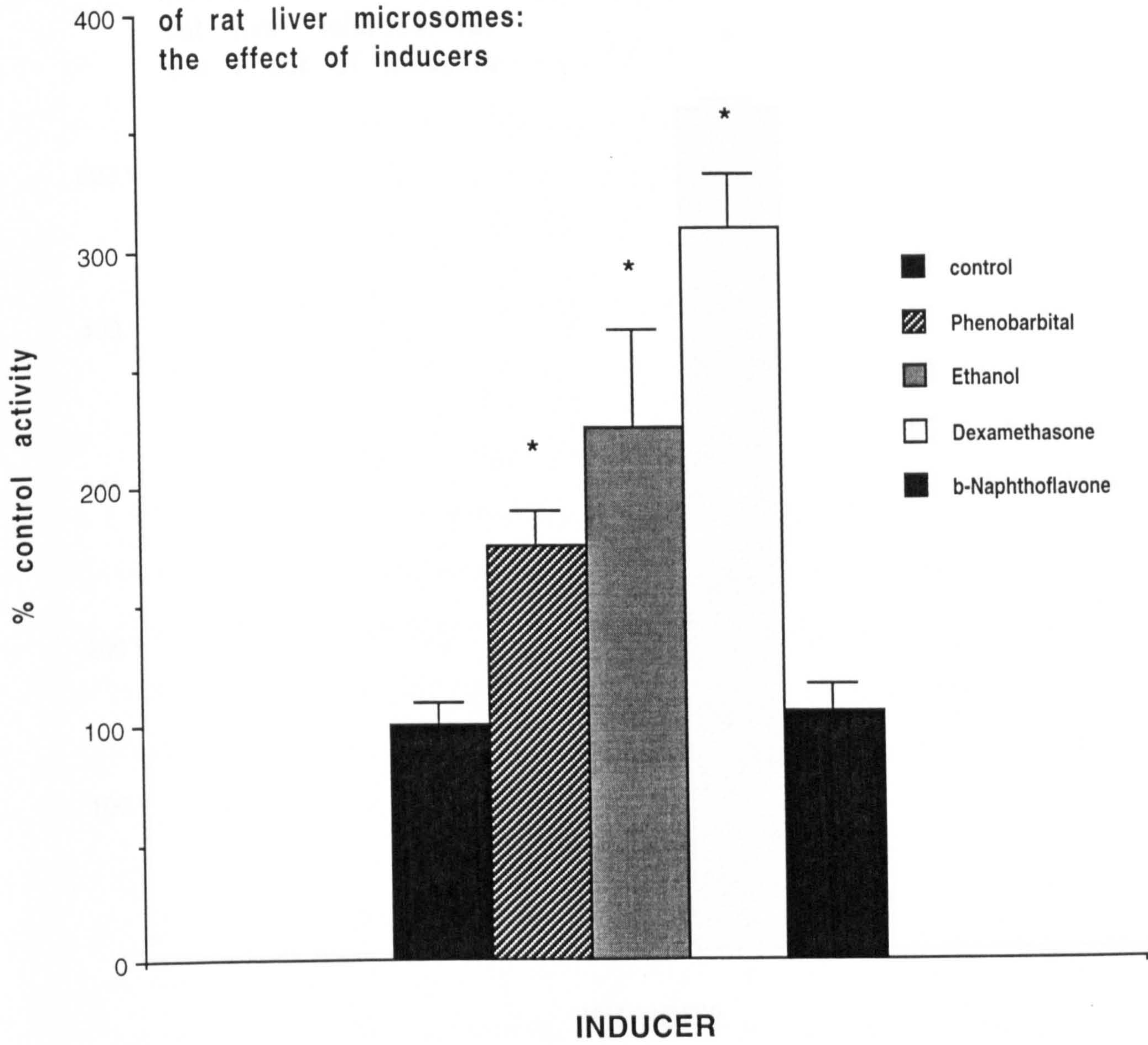
(ANOVA with modified t test)

Control (100%) activities

4.2 ± 0.33 pmoles/ min/ mg protein (Saline controls)

2.2 ± 0.08 pmoles/ min/ mg protein (Corn oil controls)

Figure 9.4
 p-Nitrophenol hydroxylase activity
 of rat liver microsomes:
 the effect of inducers

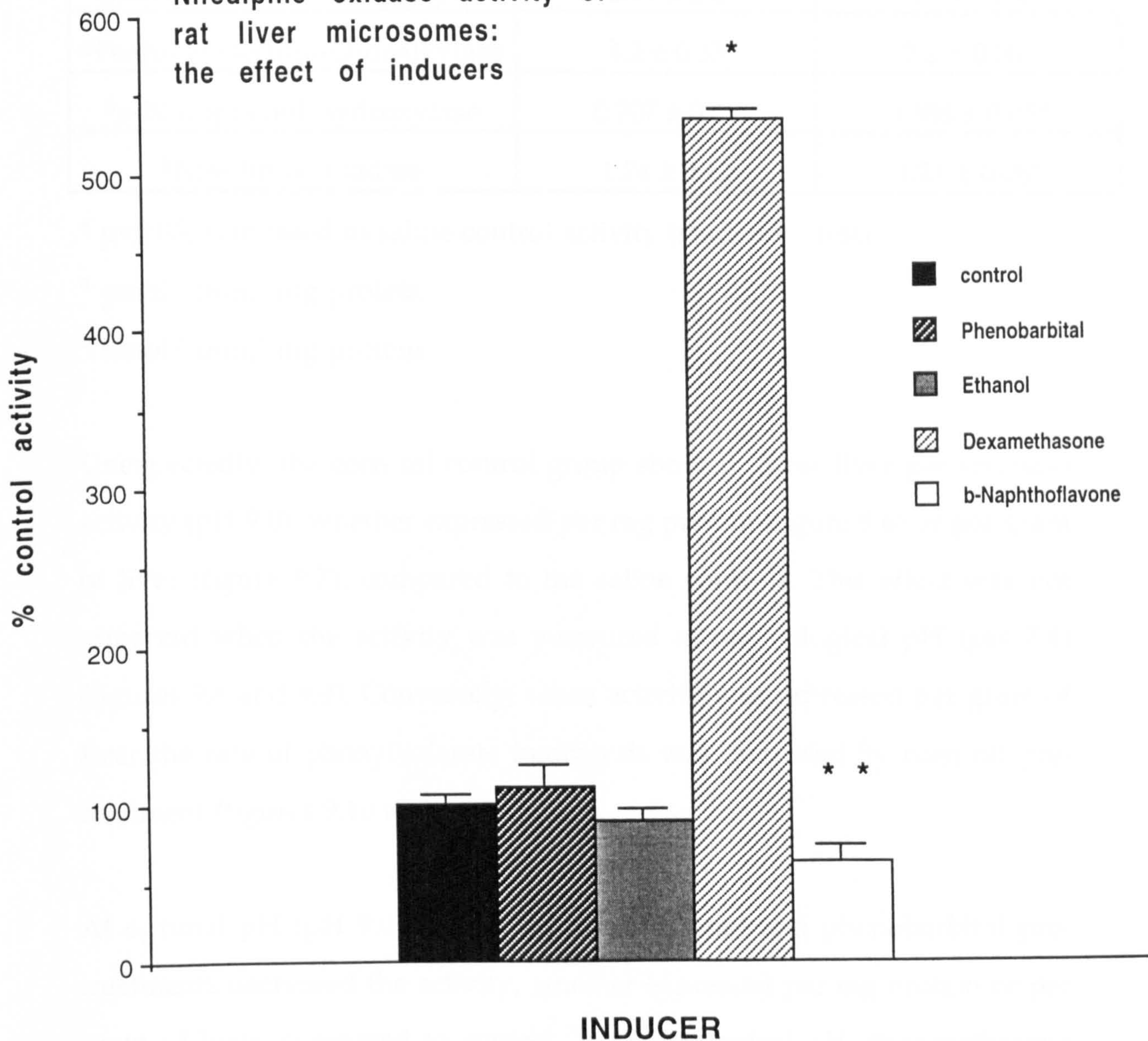


* $p < 0.05$ compared to appropriate control
 (ANOVA with modified t test)

Control (100%) activities

0.707 ± 0.07 nmoles/ min/ mg protein (Saline controls)
 0.388 ± 0.05 nmoles/ min/ mg protein (Corn oil controls)

Figure 9.5
Nifedipine oxidase activity of
rat liver microsomes:
the effect of inducers



* $p < 0.05$, higher compared to appropriate control

** $p < 0.05$, lower compared to appropriate control

(ANOVA with modified t test)

Control (100%) activities

1.74 ± 0.12 nmoles/ min/ mg protein (Saline controls)

1.21 ± 0.08 nmoles/ min/ mg protein (Corn oil controls)

Table 9.9 The effects of corn oil pre-treatment on various cytochromes P450 of rat liver. Results are the mean \pm sem for n = 5 rats.

Activity	Saline controls	Corn oil controls
^a Ethoxyresorufin -O-dealkylase	28.1 \pm 2.4	27.8 \pm 3.4
^a Pentoxyresorufin-O-dealkylase	4.2 \pm 0.33	2.2 \pm 0.08*
^b <i>p</i> -Nitrophenol hydroxylase	0.707 \pm 0.07	0.388 \pm 0.05*
^b Nifedipine oxidase	1.74 \pm 0.12	1.21 \pm 0.08*

* p<0.05, compared to saline control activity (students t-test)

^a pmol/ min/ mg protein

^b nmol/ min/ mg protein

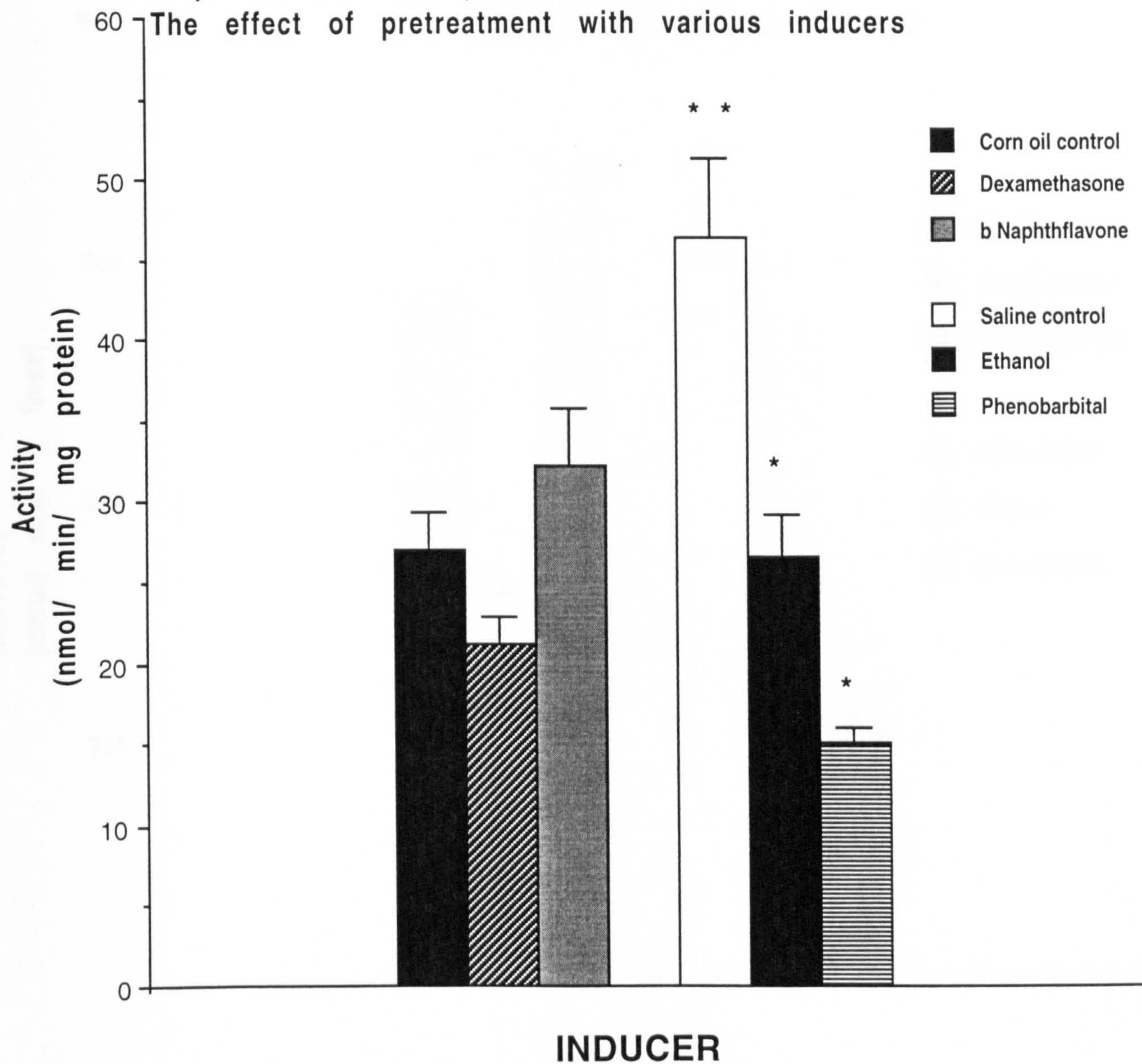
Unexpectedly, the corn oil control group showed lower liver paraoxonase activity (pH 9.0), whether expressed per mg protein (figure 9.6) or per gram of liver (figure 9.7), compared to the saline controls. This effect was not apparent when the activity was measured at physiological pH (pH 7.4) (figures 9.8 and 9.9). Conversely, when activity was expressed per gram of liver the rate of phenylvalerate hydrolysis was increased by corn oil pre-treatment (figures 9.10 and 9.11).

At optimal pH (pH 9.0) for paraoxonase, ethanol and phenobarbital pre-treatments decreased the activity, whether expressed per mg protein or per gram of liver, compared to control. At physiological pH, dexamethasone caused a decrease in paraoxonase activity (whether expressed per mg protein or per gram of liver) while phenobarbital only had this effect when activity was expressed per mg protein. Ethanol pre-treatment increased the rate of hydrolysis of paraoxon at physiological pH, but this was true only when activity was expressed per gram of liver.

Figure 9.6

Rat liver paraoxonase activity (30°C) measured at pH 9.0 with 1mM paraoxon:

The effect of pretreatment with various inducers



* $p < 0.05$, lower compared to saline control

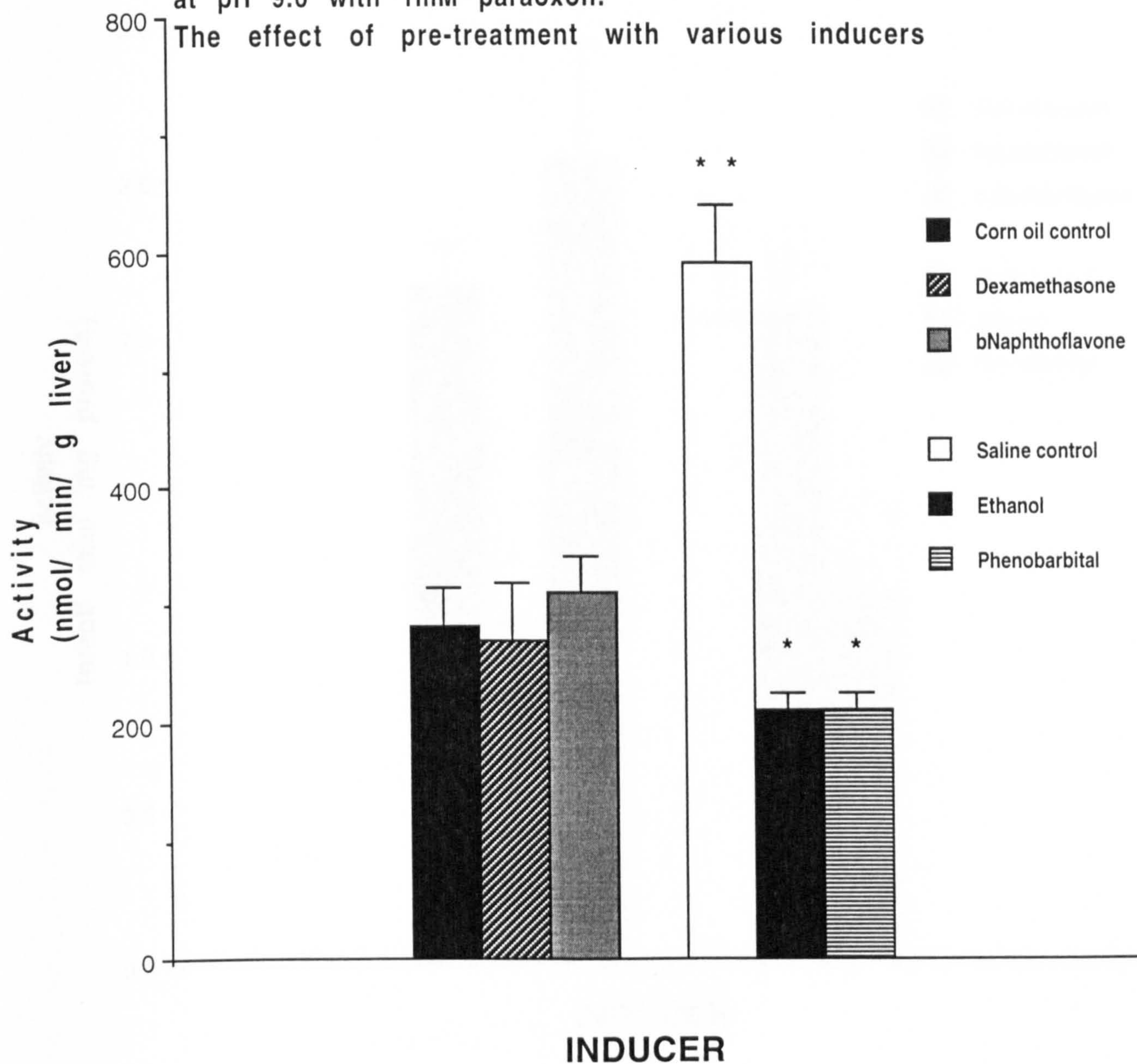
** $p < 0.05$, higher compared to corn oil control

(ANOVA with modified t test)

Figure 9.7

Rat liver paraoxonase activity (30°C) measured at pH 9.0 with 1mM paraoxon:

The effect of pre-treatment with various inducers

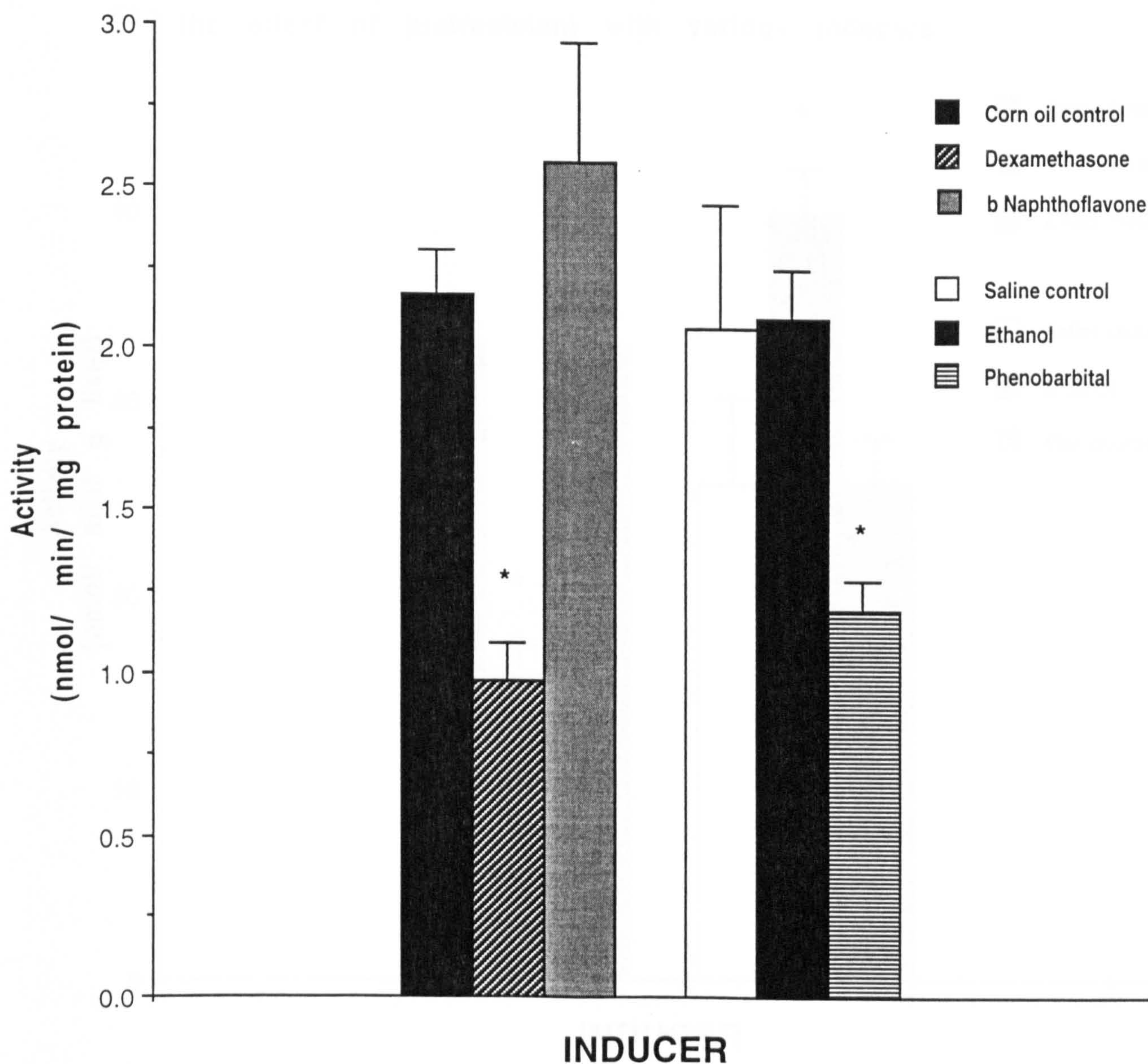


* $p < 0.05$, lower compared to saline control

** $p < 0.05$, higher compared to corn oil control

(ANOVA with modified t test)

Figure 9.8
Rat liver paraoxonase activity (30°C)
measured at pH 7.4 with 1mM paraoxon:
the effect of pretreatment with various inducers



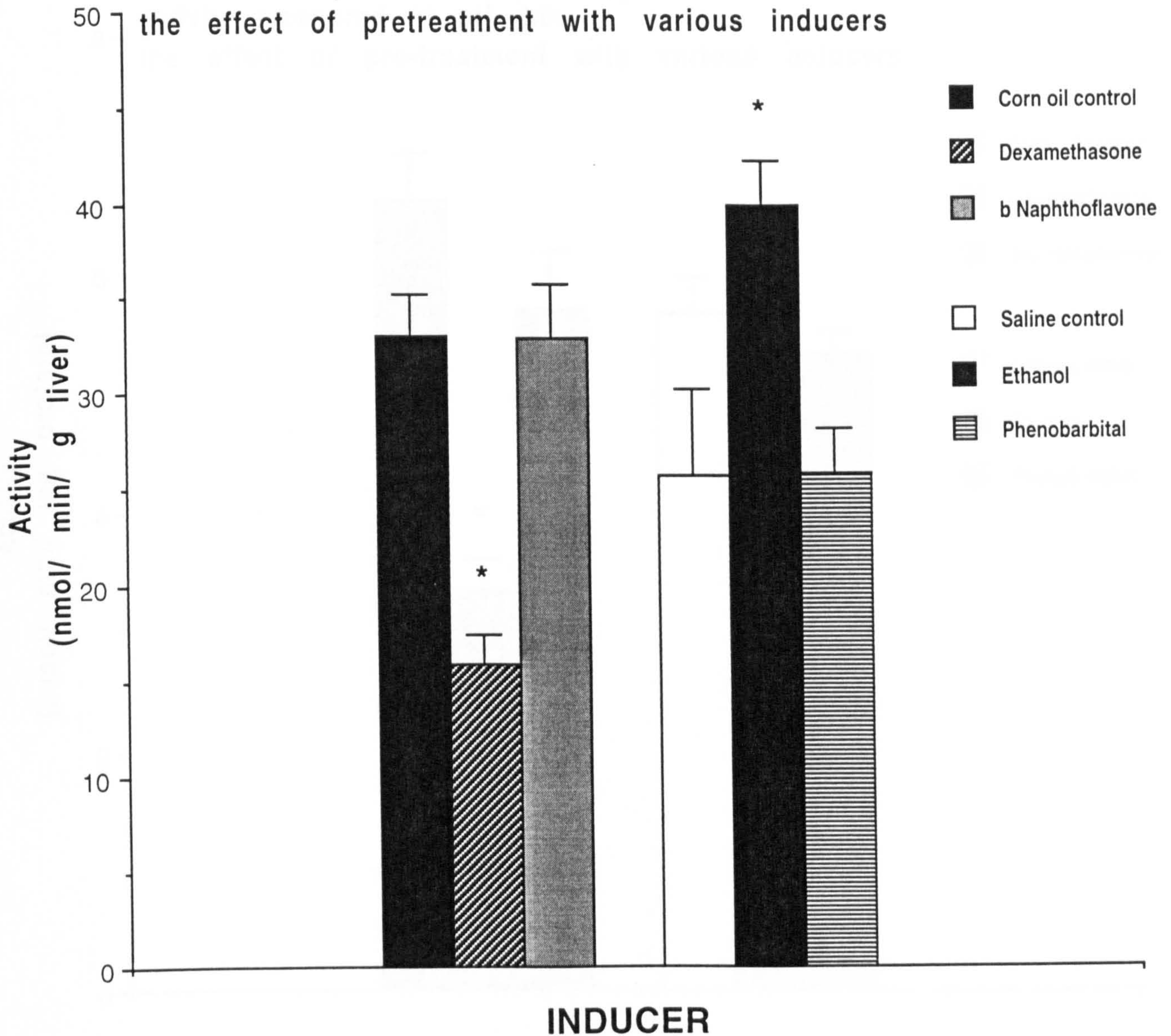
* $p < 0.05$ compared to appropriate control
(ANOVA with modified t test)

Figure 9.9

Rat liver paraoxonase activity (30°C)

measured at pH 7.4 with 1mM paraoxon:

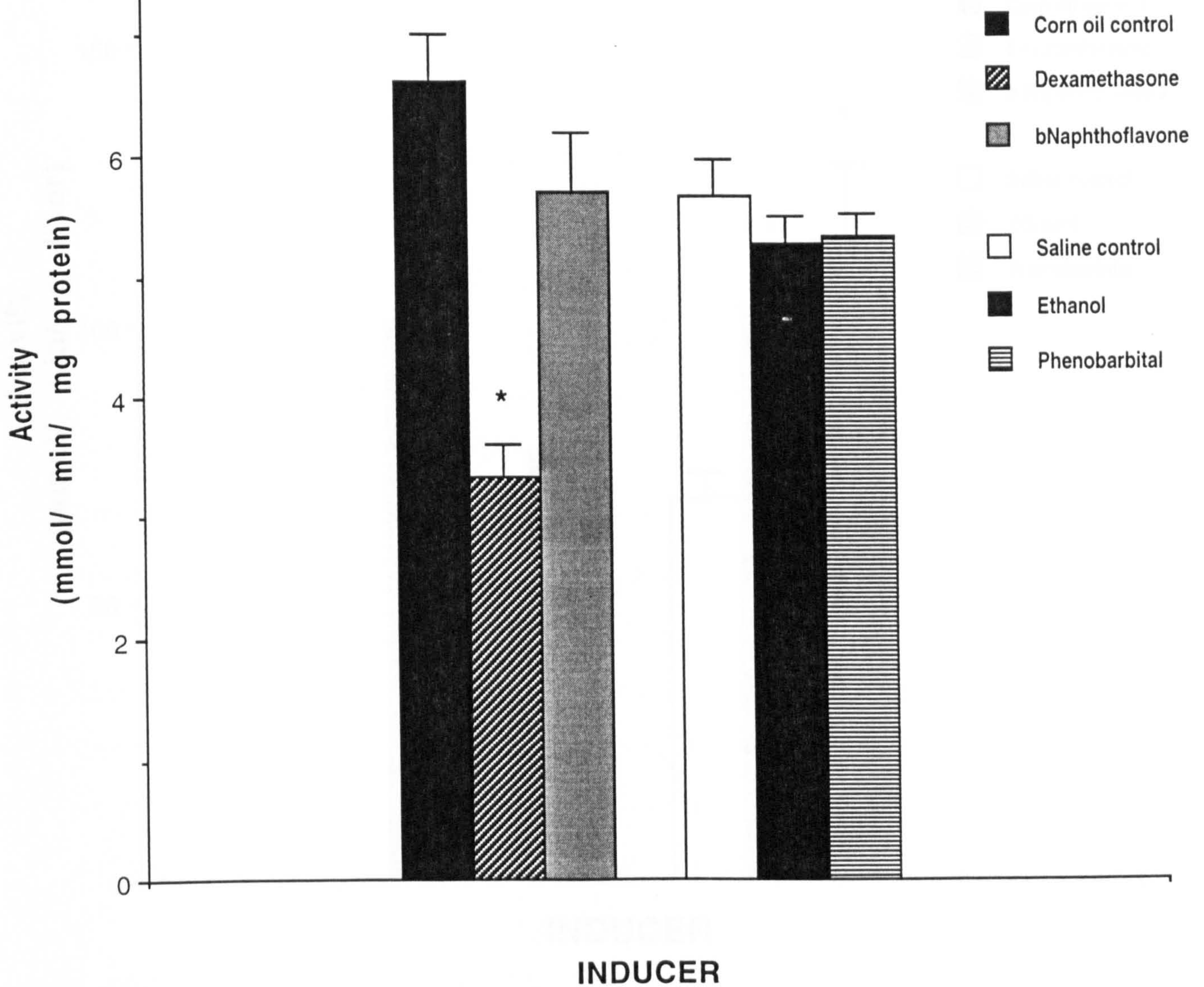
the effect of pretreatment with various inducers



* $p < 0.05$ compared to appropriate control

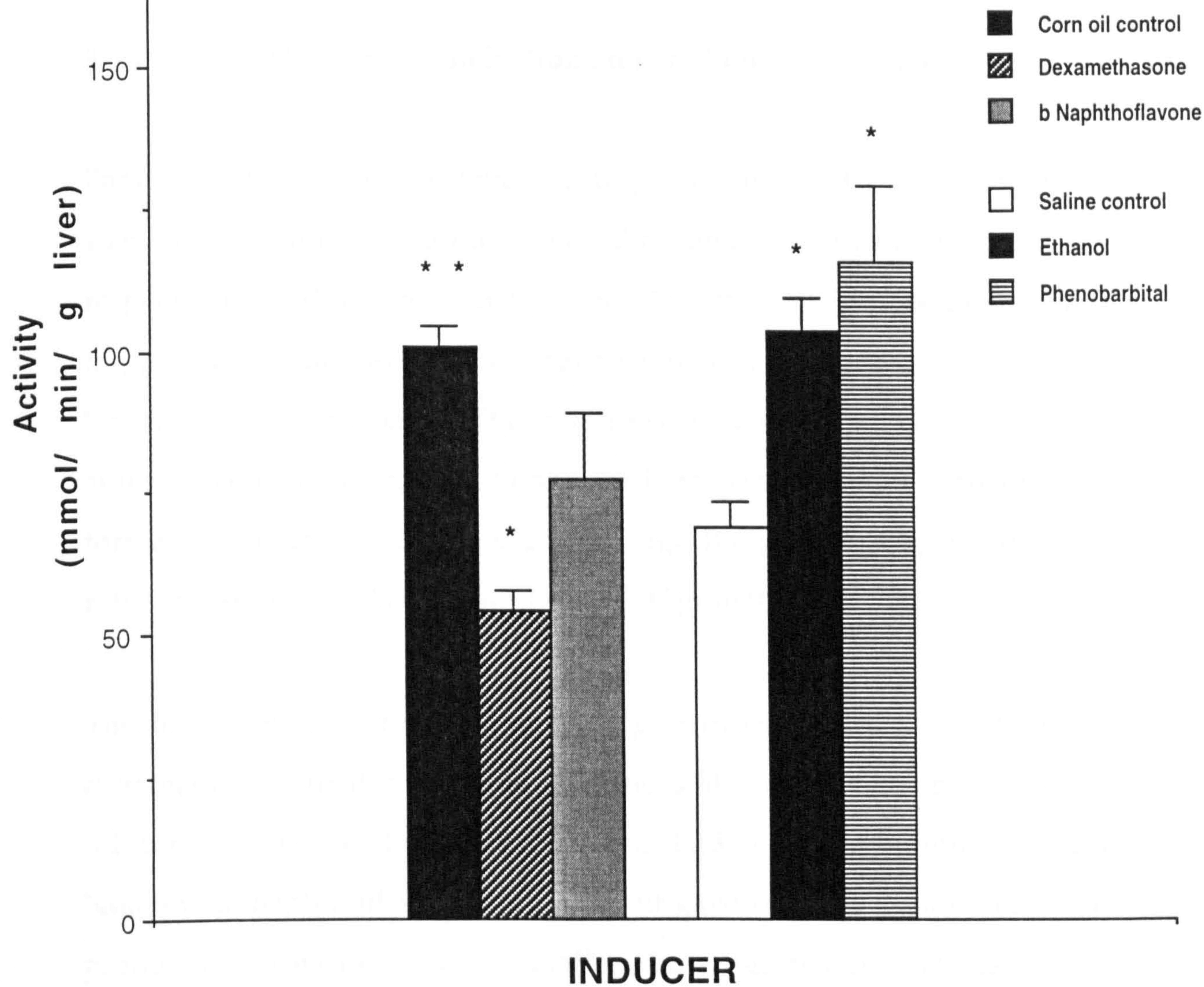
(ANOVA with modified t test)

Figure 9.10
Rat liver phenylvalerate hydrolase (1.4mM)
activity measured at pH 8.0:
the effect of pre-treatment with various inducers



* $p < 0.05$ compared to corn oil control
(ANOVA with modified t test)

Figure 9.11
 Rat liver phenylvalerate hydrolase (1.4mM)
 activity measured at pH 8.0:
 the effect of pre-treatment with various inducers



* p<0.05 compared to appropriate control
 ** p<0.05, higher compared to saline control
 (ANOVA with modified t test)

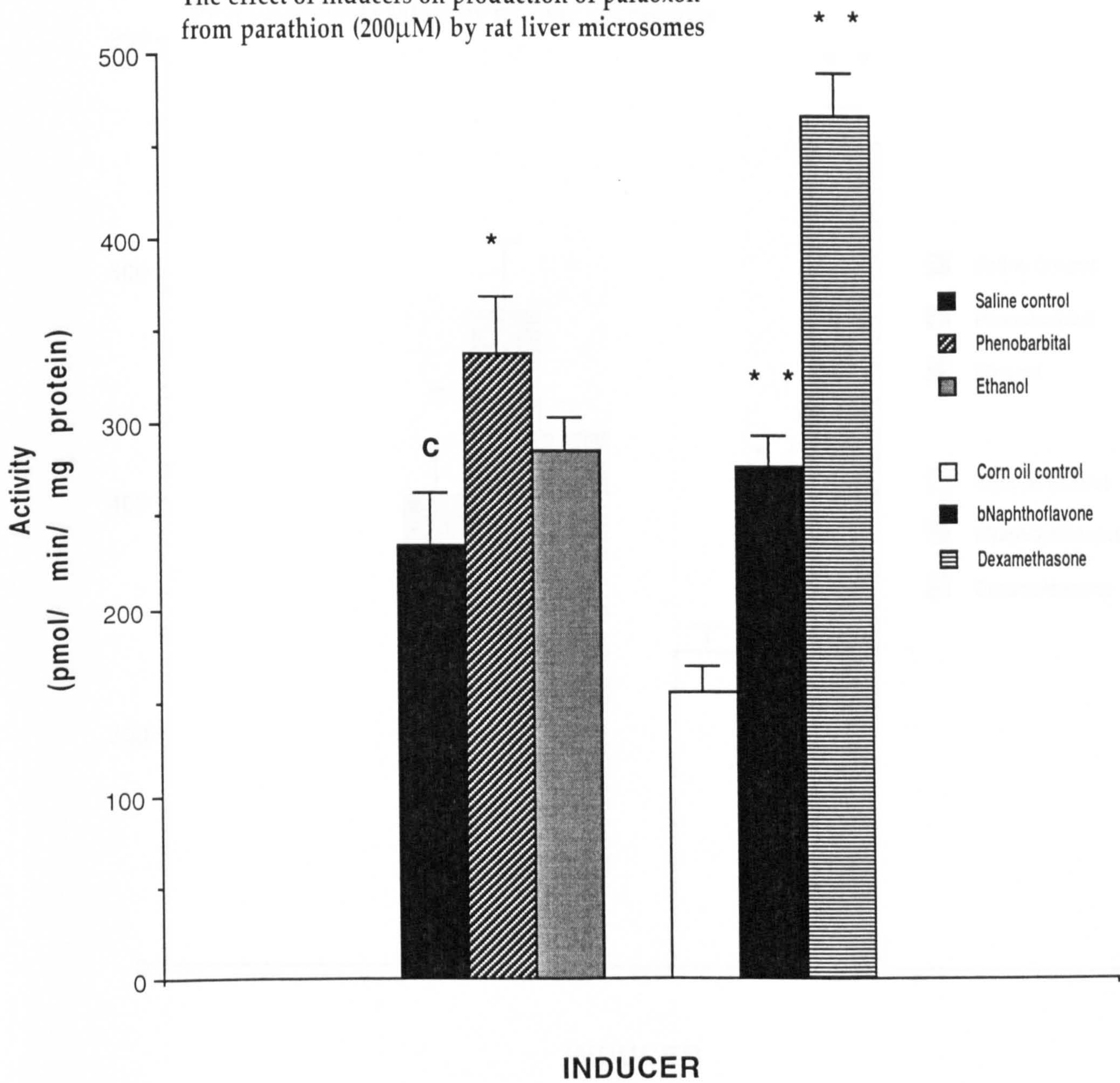
Dexamethasone pre-treatment decreased phenylvalerate hydrolase activity, whether expressed per mg protein or per gram of liver, compared to control. Ethanol and phenobarbital pre-treatments increased the rate of hydrolysis of phenylvalerate, but only when activities were expressed per gram of liver.

9.3.4 The effect of induction on parathion metabolism

Parathion (200 μ M) was metabolised to paraoxon (234.0 ± 28.1 and 155.1 ± 13.6 , pmol/ min/ mg protein for saline and corn oil-treated controls, respectively) and *p*-nitrophenol (407.8 ± 45.2 and 271.3 ± 22.4 pmol/ min/ mg protein by microsomes from saline and corn oil controls, respectively) by rat liver microsomes. The activities of saline and corn oil dosed animals were significantly different for both paraoxon and *p*-nitrophenol formation (figures 9.12 and 9.13), although the paraoxon/ *p*-nitrophenol ratio was similar for both control groups (figure 9.14).

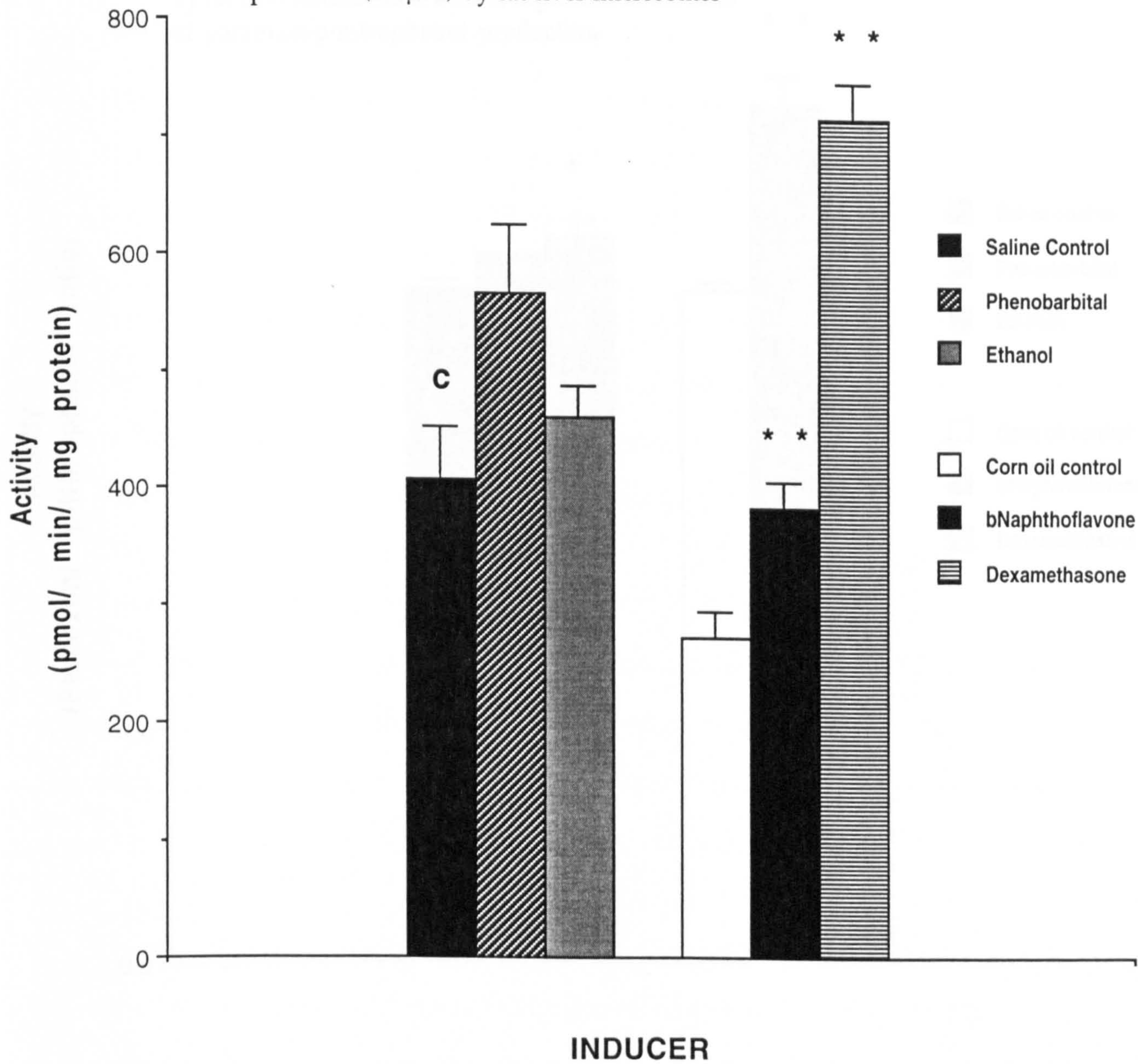
The formation of both paraoxon and *p*-nitrophenol from parathion by dexamethasone-treated livers was enhanced about 3-fold compared to corn oil controls, while the other regimens had a less pronounced effect. Notably, β -naphthoflavone pre-treatment gave rise to a 2-fold increase in paraoxon formation and a smaller, but significant, increase in *p*-nitrophenol formation. Phenobarbital pre-treatment also significantly enhanced formation of paraoxon but failed to increase *p*-nitrophenol formation ($p=0.07$). Although ethanol pre-treatment did not enhance paraoxon or *p*-nitrophenol formation individually, it did significantly increase the paraoxon/ *p*-nitrophenol ratio (figures 9.12, 9.13 and 9.14).

Figure 9.12
 The effect of inducers on production of paraoxon from parathion (200μM) by rat liver microsomes



* $p < 0.05$ compared to saline control
** $p < 0.05$ compared to corn oil control
^c $p < 0.05$, higher compared to corn oil control
(ANOVA with modified t test)

Figure 9.13
The effect of inducers on production of p-nitrophenol from parathion (200μM) by rat liver microsomes



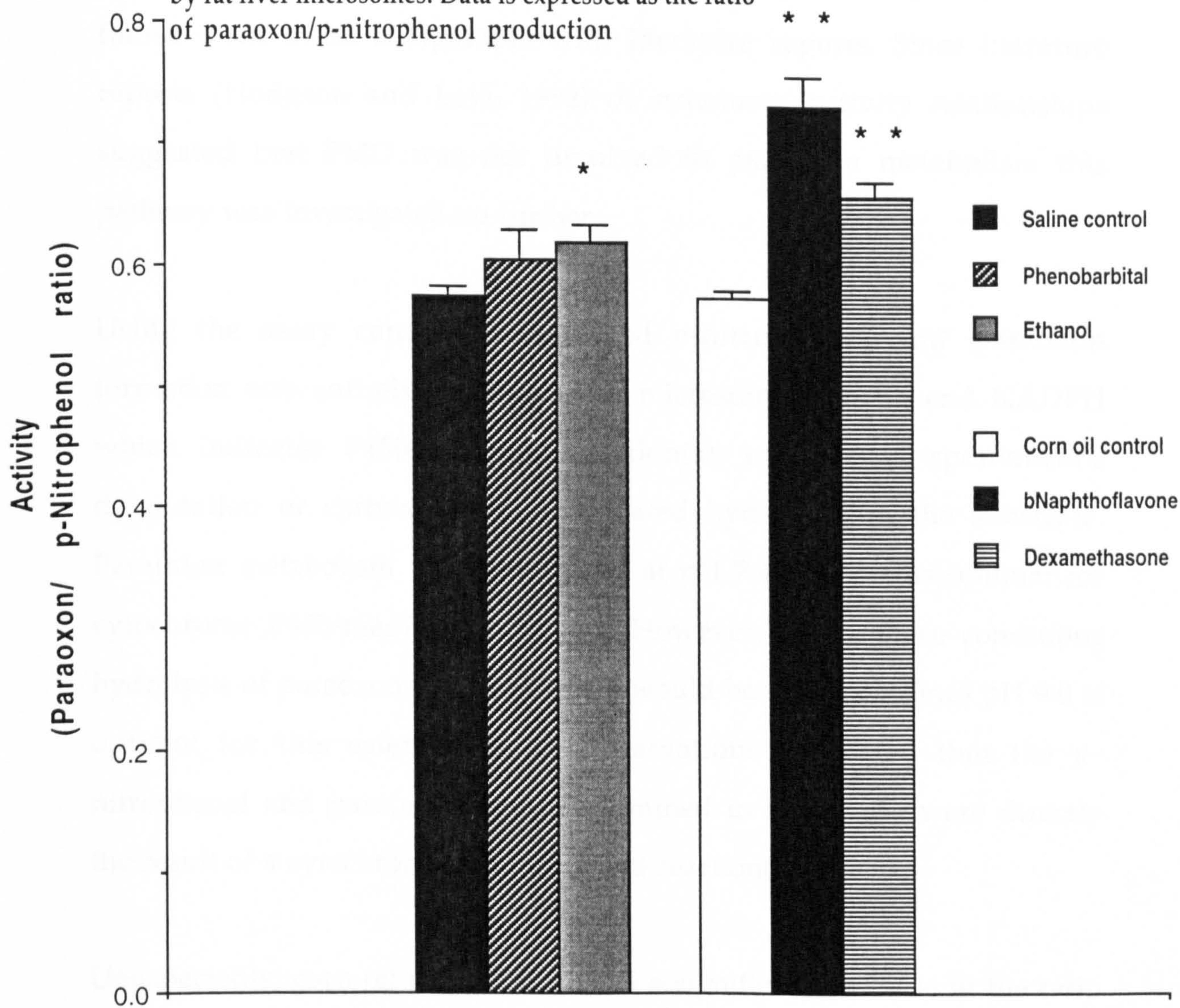
** $p < 0.05$ compared to corn oil control

c $p < 0.05$, higher compared to corn oil control

(ANOVA with modified t test)

Figure 9.14

The effect of inducers on the production of paraoxon and p-nitrophenol from parathion by rat liver microsomes. Data is expressed as the ratio of paraoxon/p-nitrophenol production



* $p < 0.05$ compared to saline control

** $p < 0.05$ compared to corn oil control

(ANOVA with modified t test)

Initial studies defined control activities for the cytochromes P450 and esterases which may be involved in the metabolism of parathion and found them to be comparable with literature reports. Since literature reports (Hodgson and Levi, 1992) of structure/ activity relationships suggested that FMO was not involved in parathion metabolism this pathway was investigated no further.

Using the assay conditions described *p*-nitrophenol and paraoxon formation was entirely dependent on microsomal protein and NADPH which indicates P450-mediated reaction(s), rather than spontaneous degradation or carboxylesterase-mediated hydrolysis of the substrate. Parathion metabolism was determined at pH 7.4 which was optimal for cytochrome P450-mediated reactions. However, under these conditions hydrolysis of paraoxon by paraoxonase would be negligible since pH 9.0 is optimal for this esterase. These observations suggested that the *p*-nitrophenol and paraoxon levels determined in this study were directly the result of a cytochrome P450-mediated reaction(s).

Unexpectedly, corn oil treatment caused a significant decrease in the ratio of liver weight to body weight compared to saline controls. Additionally, recovery of microsomal protein and P450 content of the corn oil-treated livers was significantly higher than in the saline control animals. This suggested induction of membrane proteins, including P450s, by corn oil with a concomitant decrease in liver weight. Other researchers have observed similar changes following treatment with corn oil. For example, a recent study by Kojo and co-workers (1996) demonstrated significantly lowered catalytic activities for the P450 2A and 2B subfamilies 24h after

corn oil treatment compared to untreated controls and speculated that this effect may be related to disturbances in fatty acyl homeostasis which can influence the function of membrane-bound proteins, such as the P450s (Kojo *et al*, 1996). This may be an explanation for the results seen in the present study and demonstrates the need for inclusion of appropriate vehicle controls when conducting such investigations. It was assumed that the effects of the inducers were so great compared to the effects of corn oil that the outcome of these compounds may be considered distinct from those of the oil.

Ethanol treatment caused a significant decrease in liver weight and in the ratio of liver weight to body weight compared to saline controls. Microsomal protein recovery was increased but, since P450 content remained unchanged, presumably the level of other membrane proteins were preferentially increased with this treatment. Conversely, dexamethasone and β -naphthoflavone treatment increased the ratio of liver weight to body weight compared to corn oil controls which relates to the observation that both these compounds gave rise to a mottled appearance to the livers. Dexamethasone, but not β -naphthoflavone treatment, also caused an increase in microsomal protein recovery and total P450 content. Although phenobarbital treatment did not significantly increase the ratio of liver weight to body weight, it did significantly increase microsomal protein recovery and total P450 content compared to saline controls.

The poor level of enhancement of *p*-nitrophenol hydroxylase activity by ethanol corroborates the observation that the total P450 content of these livers was not increased following this treatment. A regimen involving ethanol treatment by i.p. injection may have been a more efficient method

to induce CYP2E1. Low level (approximately equivalent to the plasma ethanol concentration after two units of alcoholic beverage) ethanol exposure has been shown to induce CYP3A (and CYP2E1) in human hepatocyte cultures *in vitro* (Kostrubsky *et al*, 1995). In this study, however, pre-treatment with ethanol had no effect on any of the CYPs measured, other than CYP2E1.

In addition to the expected induction of pentoxyresorufin-*O*-dealkylation by phenobarbital (approximately 50-fold), this pre-treatment also enhanced *p*-nitrophenol hydroxylation (CYP2E1), by about 2-fold. Barbiturates, such as phenobarbital, are well known powerful inducers of cytochromes P450 2B1/2 (as well as other enzyme systems) in rat liver (Parkinson, 1996).

β -Naphthoflavone pre-treatment, as expected, induced the rate of ethoxyresorufin-*O*-dealkylation (CYP1A), by about 50-fold, but also enhanced the *O*-dealkylation of pentoxyresorufin (approximately 5-fold). Conversely, this pre-treatment had no effect on *p*-nitrophenol hydroxylation and significantly lowered nifedipine oxidation. Treatment of rats with β -naphthoflavone is known to give rise to prominent induction of CYP1A1, which is virtually undetectable in control liver, and also probably of the constitutive isoform, CYP1A2. Enhanced pentoxyresorufin-*O*-dealkylation following β -naphthoflavone treatment has also been observed by Burke *et al* (1994) who confirmed that pentoxyresorufin is mainly metabolised by the CYP1A subfamily in β -naphthoflavone-induced rat liver microsomes.

The induction of nifedipine oxidase activity was mediated exclusively by dexamethasone and therefore metabolism of this substrate by these induced livers would have principally involved CYP3A1 (the inducible

CYP3A male rat liver isoform), since the constitutive CYP3A2 isoform is not affected by this inducer (Lindamood, 1991). The mechanism of dexamethasone induction involves transcriptional activation and mRNA stabilisation (Soucek and Gut, 1992). Other researchers (Soucek and Gut, 1992) have suggested that CYP3A1 is also inducible by phenobarbital, although this was not indicated in the present study since nifedipine oxidation was not increased by phenobarbital pre-treatment. This anomaly may result from the choice of substrate for CYP3A activity since it is known that nifedipine is metabolised by CYP3A2 and CYP2C11 in control male rat liver (Guengerich *et al*, 1986) and paradoxically CYP2C11, the isoform of highest constitutive activity in male rat liver, is decreased by phenobarbital pre-treatment (Parkinson, 1996).

Dexamethasone pre-treatment also enhanced ethoxyresorufin-O-dealkylase and pentoxyresorufin-O-dealkylase activities (3-fold and 15-fold, respectively) and, unexpectedly, induced *p*-nitrophenol hydroxylase activity to a greater extent (3-fold) than that observed by ethanol (2-fold). Induction of CYP2B (pentoxyresorufin-O-dealkylase) by dexamethasone has been well documented (Rao *et al*, 1990) although the mechanism of this induction is not yet understood.

The esterase study showed that the rate of phenylvalerate hydrolysis was increased by corn oil pre-treatment when activity was expressed per gram of liver. However, the corn oil vehicle had no appreciable effect on paraoxonase activity at physiological pH, although activity was decreased at pH 9.0 compared to saline controls. The observed increase in phenylvalerate hydrolysis was contrary to a report by Watson *et al* (1994) which considered corn oil treatment responsible for lowered microsomal carboxylesterase activity compared to untreated controls. These authors

suggested that altered membrane properties may have affected the configuration of the carboxylesterase molecule and so caused the lowered activity.

For several reasons measurement of esterase activity at physiological pH was more appropriate than determinations made at optimal pH. Firstly, data obtained at pH 7.4 would be more applicable to the situation *in vivo*, even though the concentration of paraoxon (in the case of paraoxonase measurement) used *in vitro* would be lethal if encountered *in vivo*. Although esterases often have non-physiological pH optima, this is not true of the cytochromes P450, which have a requirement for approximately pH 7.4. It follows that, when investigating metabolism of phosphorothioates in which P450 and esterase pathways function in parallel, the *in vitro* system utilised should be maintained at physiological pH.

At physiological pH, dexamethasone, and possibly phenobarbital (when activity was expressed per mg protein), decreased paraoxonase activity. Taken in isolation, this suggested a decreased capacity to detoxify toxic oxons, such as paraoxon, following co-administration of these drugs. McCracken *et al* (1993), however, demonstrated that hepatic microsomal paraoxonase activity was enhanced approximately two-fold by phenobarbital pre-treatment, the reasons for the anomaly between the two sets of data are unknown. Conversely, ethanol pre-treatment increased the rate of hydrolysis of paraoxon (expressed per gram of liver) which, if taken *per se*, indicates enhanced detoxification capacity.

When measurements were made at pH 9.0, which was optimal for paraoxonase activity, ethanol and phenobarbital pre-treatments were seen

to decrease the rate of hydrolysis of paraoxon. However, the decrease in paraoxonase activity by dexamethasone observed at physiological pH, was not seen at pH 9.0, although this result may have been confounded by the inhibitory effects of corn oil on the control group at this pH.

Dexamethasone pre-treatment decreased carboxylesterase and cholinesterase activity, whether activity was expressed per mg protein or per gram of liver. Conversely, ethanol- and phenobarbital-treated livers had enhanced hydrolytic activity towards phenylvalerate, but only when expressed per gram of liver. Several research groups (eg Chambers *et al*, 1994) have also observed an increase in carboxylesterase activity following phenobarbital treatment, however the effect of ethanol on these esterases has not been studied. These data suggest that administration of ethanol or phenobarbital may increase hepatic capacity to detoxify oxons by this pathway which may contribute to the attenuation of toxicity following exposure to phosphorothioates.

The study of parathion metabolism suggested the participation of CYP3A in the reaction since paraoxon and *p*-nitrophenol formation was increased by dexamethasone in parallel with nifedipine oxidation (a CYP3A-mediated reaction). Cytochromes P450 1A1/2 also had the capacity to metabolise parathion following induction (with β -naphthoflavone), although they are minor isoforms in untreated animals. The paraoxon/ *p*-nitrophenol ratio was also significantly perturbed following both dexamethasone and β -naphthoflavone pre-treatments, possibly suggesting that the metabolites were not derived from a common intermediate or, perhaps, that further metabolism had taken place following induction. Interestingly, a recent study by Zerilli *et al* (1997) suggested that CYP3A was

responsible for about 50% of *p*-nitrophenol hydroxylation in liver microsomes from dexamethasone pre-treated rats.

An investigation by O'Shaughnessy and Sultatos (1995) of the effects *in vitro* of ethanol on parathion metabolism in the mouse showed that this compound did not directly affect the P450-mediated metabolism of this phosphorothioate. However, ethanol was demonstrated to antagonise its acute toxicity *in vivo*. The authors suggested that this attenuation of toxicity may have resulted from a reduced availability of molecular oxygen which decreased the P450-mediated activation of parathion *in vivo*. The data obtained in the present study for ethanol was puzzling since neither paraoxon nor *p*-nitrophenol formation was increased by ethanol pre-treatment, yet the paraoxon/ *p*-nitrophenol ratio was significantly perturbed. This indicates that CYP2E1 was not involved in the activation of parathion but, perhaps, increased levels of this P450 isoform was involved in the further metabolism of *p*-nitrophenol.

The increase in paraoxon formation following phenobarbital treatment suggested that CYP2B may also play a role in parathion activation in the induced animal, although this compound has also been well documented to also induce CYP3A in the rat (Okey *et al*, 1990). Induction of CYP3A by phenobarbital was not confirmed in the present study since nifedipine oxidation was not increased. This result, however, may be an anomaly and due to the choice of substrate for CYP3A activity, rather than a lack of induction of CYP3A. *p*-Nitrophenol production was not significantly increased by phenobarbital pre-treatment and therefore did not parallel the enhanced paraoxon formation. However, the variability of these data were large and influenced the significance of the result. Alternatively, increased

levels of CYP2E1 may have been involved in further metabolism of *p*-nitrophenol.

In conclusion, these data demonstrated that the respective P450s were effectively enhanced by the induction regimen. However, it was difficult to pinpoint the P450 isoform(s) responsible for the metabolism of parathion from the induction study alone, but P450s 3A, 1A and possibly 2B were implicated in the induced animal. Interpretation of data was confounded by the fact that the substrates used were not specific for a particular isoform and that induction by a particular compound was not specific to an individual P450 isoform.

Chapter 10

Chapter 10 Rat inhibition studies *in vitro*

10.1 Introduction

It was clear from the induction studies that parathion could be metabolised by a P450-mediated reaction(s) which involved the cytochromes P450 3A, 1A and possibly 2B in induced rat liver microsomes. It was also shown that the toxic oxon, paraoxon, was detoxified by several esterase pathways. In this chapter the ability of control rat liver microsomes to metabolise parathion was modulated by pre-incubation with various P450 inhibitors, *in vitro*. The aim of these studies was therefore to indicate the constitutive P450 isoform(s) involved in parathion metabolism. The inhibitors used in the study were chosen from the literature on the basis of effects on human P450 isoforms as little information was available for the rat orthologous forms.

Since the induction study strongly implicated CYP3A in parathion metabolism, several inhibitors of this subfamily were used, namely naringenin, troleandomycin, quercetin and ketoconazole (Guengerich and Kim, 1990; Maurice *et al*, 1992 and Guengerich, 1990). Quinidine is an inhibitor of the human P450 2D6 subfamily (Guengerich *et al*, 1986) and α -naphthoflavone inhibited P450 1A reactions (Shimada and Guengerich, 1991). Diethyldithiocarbamate (DEDIC) is an inhibitor of cytochromes P450 2E1 and 2A6 in man (Yamazaki *et al*, 1992), tolbutamide is a substrate for human P450 2C9 (Gonzales, 1992) and sulphaphenazole is an inhibitor of P450 2C9 (Baldwin *et al*, 1995). Metyrapone is a non-specific inhibitor of P450 2B.

Naringenin, quercetin and ketoconazole are reversible inhibitors of P450 3A and troleandomycin is a mechanism-based inhibitor of this P450 subfamily. Quinidine is a reversible inhibitor of P450 2D6 and a substrate for P450 3A at concentrations above 100µM. Diethyldithiocarbamate is a mechanism-based inhibitor of P450 2E1 and α-naphthoflavone, which was used in this study as an inhibitor of P450 1A, has also been shown to enhance some P450 3A reactions (Shou *et al*, 1994).

Table 10.1 A list of the inhibitors/substrates used in the study and cytochromes P450 involved.

Inhibitor/ Substrate	P450 affected
Naringenin	3A
*Troleandomycin	3A
Quercetin	3A
Ketoconazole	3A
Quinidine	2D6
Metyrapone	2B
α-Naphthoflavone	1A
*Diethyldithiocarbamate	2E1
@Tolbutamide	2C9
Sulphaphenazole	2C9

* mechanism-based inhibitor

@ substrate for 2C9

Initial studies in this chapter determined the inhibition profile of nifedipine oxidation, the chosen marker reaction for CYP3A activity, with the various compounds *in vitro*. This was then compared to the profile for inhibition of parathion (20µM and 200µM) metabolism in order to indicate the constitutive P450 isoform(s) involved.

10.2 Methods

Liver microsomes were prepared from 3 male Wistar rats weighing approximately 250g by the method described in chapter 7. The microsomal protein concentration of the preparations was determined by the method of Lowry adapted by Peterson (1977) using bovine serum albumin as standard, as described in chapter 7. Biochemical methods for nifedipine oxidation and parathion metabolism are described in chapter 8. Activities for each of the inhibitor treatments were compared to appropriate vehicle control measurements and percentage change in enzyme levels calculated.

10.2.1 Inhibition of nifedipine oxidation

Inhibitors

Naringenin, troleandomycin and ketoconazole were dissolved in methanol, and quercetin and quinidine in dimethylsulphoxide (DMSO) to give 20mM stock solutions. The naringenin and troleandomycin stock solutions were then diluted in methanol to give 8mM solutions and ketoconazole stock diluted in methanol to give a 1mM solution.

Inhibitors (5 μ l) or vehicle (5 μ l DMSO or methanol, control) were added to incubation tubes containing NADPH (0.6mg) and microsomal protein (approx. 0.4mg) made up to 1ml with phosphate buffer (pH 7.4). This gave final inhibitor concentrations of 100 μ M (20mM stocks, n=3 rats), 40 μ M (8mM stocks, n=2 rats) and 5 μ M (1mM stock, n=2 rats). The tubes were pre-incubated with inhibitors or vehicle for 10min prior to starting the reaction by the addition of nifedipine (5 μ M and 100 μ M, final concentration). The reaction was terminated after 20min by addition of

400µl Tris buffer (pH 9.0) and formation of M1 determined by GC with electron capture detection.

10.2.2 Inhibition of parathion metabolism

Inhibitors

Naringenin, ketoconazole, metyrapone, diethyldithiocarbamate and tolbutamide were dissolved in methanol, and α -naphthoflavone in dimethylsulphoxide (DMSO) to give 100mM stock solutions, then 1: 10 dilutions of each were prepared to give 10mM stocks. Troleandomycin was dissolved in methanol, and quercetin and quinidine in DMSO to give 10mM working stock solutions. Similarly, sulphaphenazole was dissolved in methanol to give a 10mM stock solution, this was diluted 1: 10 to give a 1mM working stock.

Inhibitors (5µl) or vehicle (5µl DMSO or methanol, control) were added to incubation tubes containing NADPH (0.3mg) and microsomal protein (approx. 0.3mg) made up to 0.5ml with Tris/ calcium buffer (pH 7.4). This gave final inhibitor concentrations of 1mM (100mM stocks, n=3 rats), 100µM (10mM stocks, n=2 rats) and 10µM (1mM stock, n=3 rats). The tubes were pre-incubated with inhibitors or vehicle for 10min prior to starting the reaction by the addition of parathion (5µM [or 10µM sulphaphenazole], 20µM and 200µM, final concentration). The reaction was terminated after 10min (2.5min for 10µM sulphaphenazole/ 5µM parathion) by addition of 0.5ml perchloric acid (20%, w/v) and formation of *p*-nitrophenol and paraoxon determined by HPLC with u.v. detection.

10.3 Results

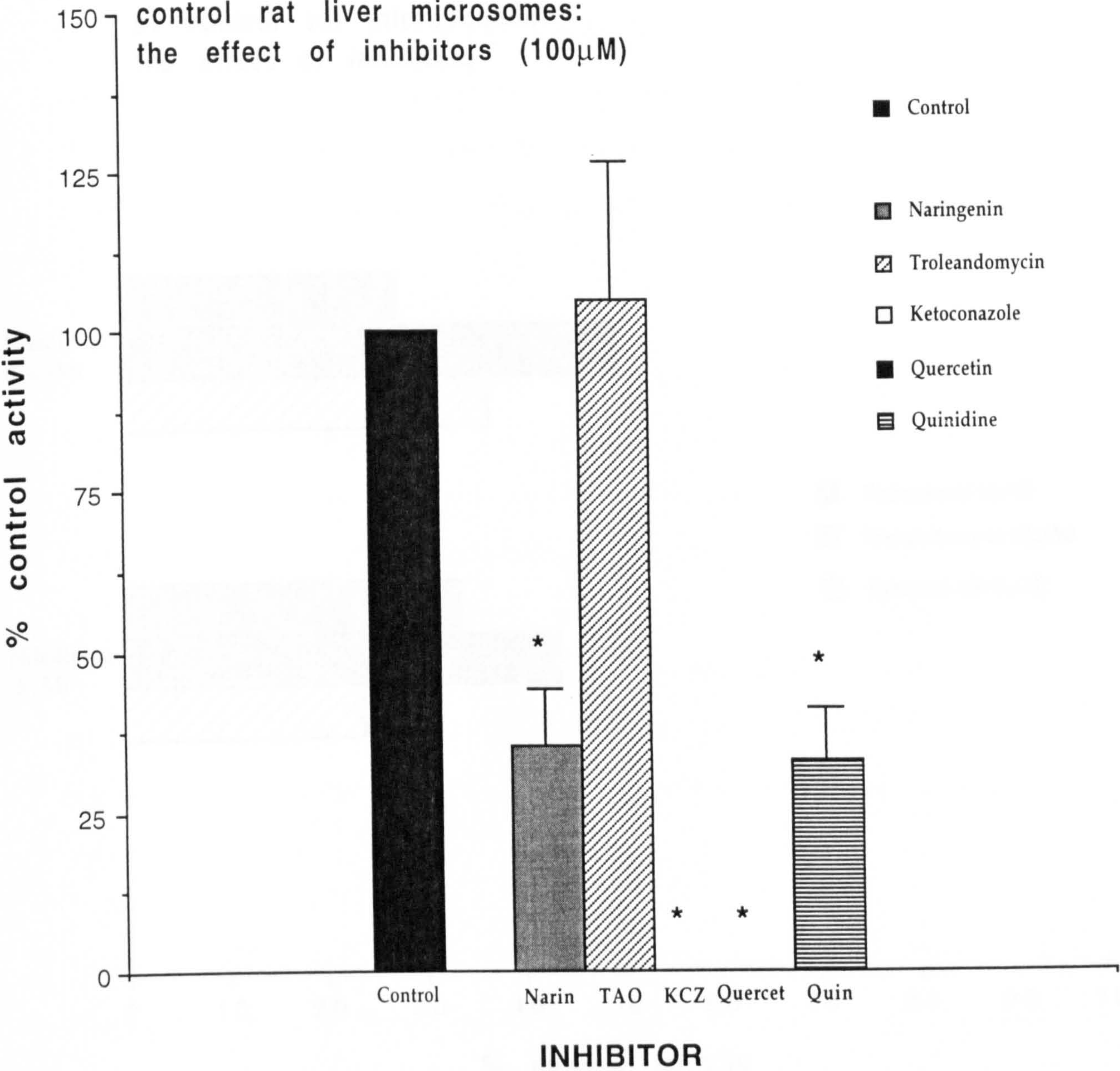
Individual data for inhibition of nifedipine oxidation and parathion metabolism are given in appendix I.

Concerning nifedipine oxidation, initial studies with inhibitors (100 μ M) and a saturating nifedipine concentration (100 μ M) (figure 10.1) showed significantly reduced (by approximately 60%) formation of M1 following the inclusion of naringenin or quinidine in the incubation. The inclusion of ketoconazole or quercetin gave rise to complete inhibition of the reaction while troleandomycin had no effect.

Later studies used naringenin (40 μ M), troleandomycin (40 μ M) and ketoconazole (5 μ M) with two concentrations of nifedipine, 5 μ M and 100 μ M (figure 10.2). The profile of inhibition was similar for 5 μ M and 100 μ M nifedipine and, although no statistical information was available (n=2 rats), the greatest inhibition was seen using naringenin (approximately 70%) and ketoconazole (approximately 65%), while troleandomycin produced approximately 50% inhibition.

With reference to parathion (200 μ M) inhibition and inhibitors (1mM), formation of paraoxon (figure 10.3) and *p*-nitrophenol (figure 10.4) was significantly lower following pre-incubation with naringenin and ketoconazole (by approximately 50% and 20%, respectively). Activity following pre-incubation with metyrapone, α -naphthoflavone and tolbutamide was not significantly different from control, while inclusion of diethyldithiocarbamate in the incubation significantly increased activity.

Figure 10.1
Nifedipine oxidase (100μM) activity of
control rat liver microsomes:
the effect of inhibitors (100μM)



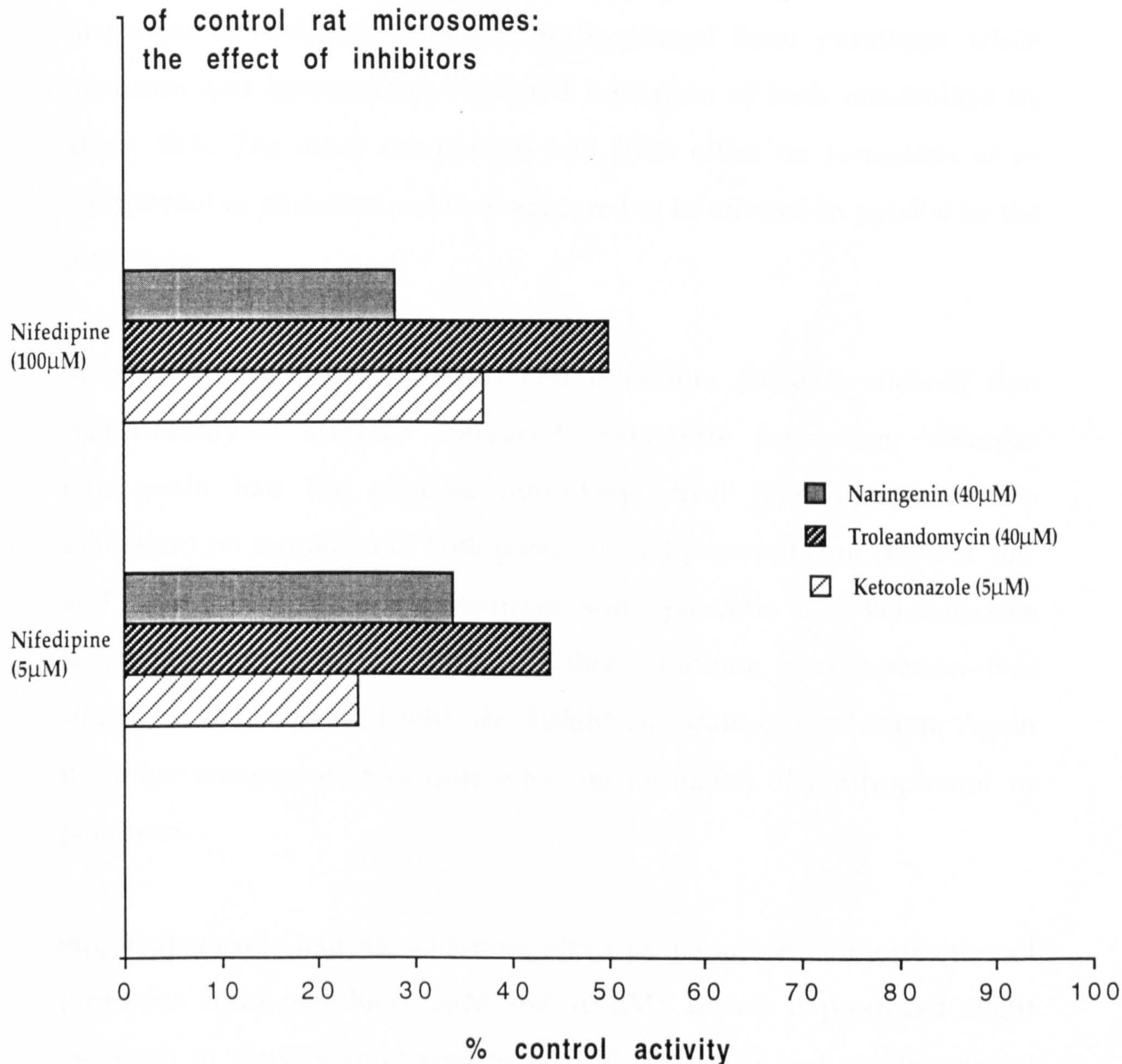
* p<0.05 compared to control activity (Students t test)

Values are the mean ± sem of n=3 rat liver microsomes
(triplicate determinations)

Individual values are given in appendix I

Figure 10.2

Nifedipine oxidase ($5\mu\text{M}$ and $100\mu\text{M}$) activity
of control rat microsomes:
the effect of inhibitors



Values are the mean of $n=2$ rat liver microsomes
(triplicate determinations)

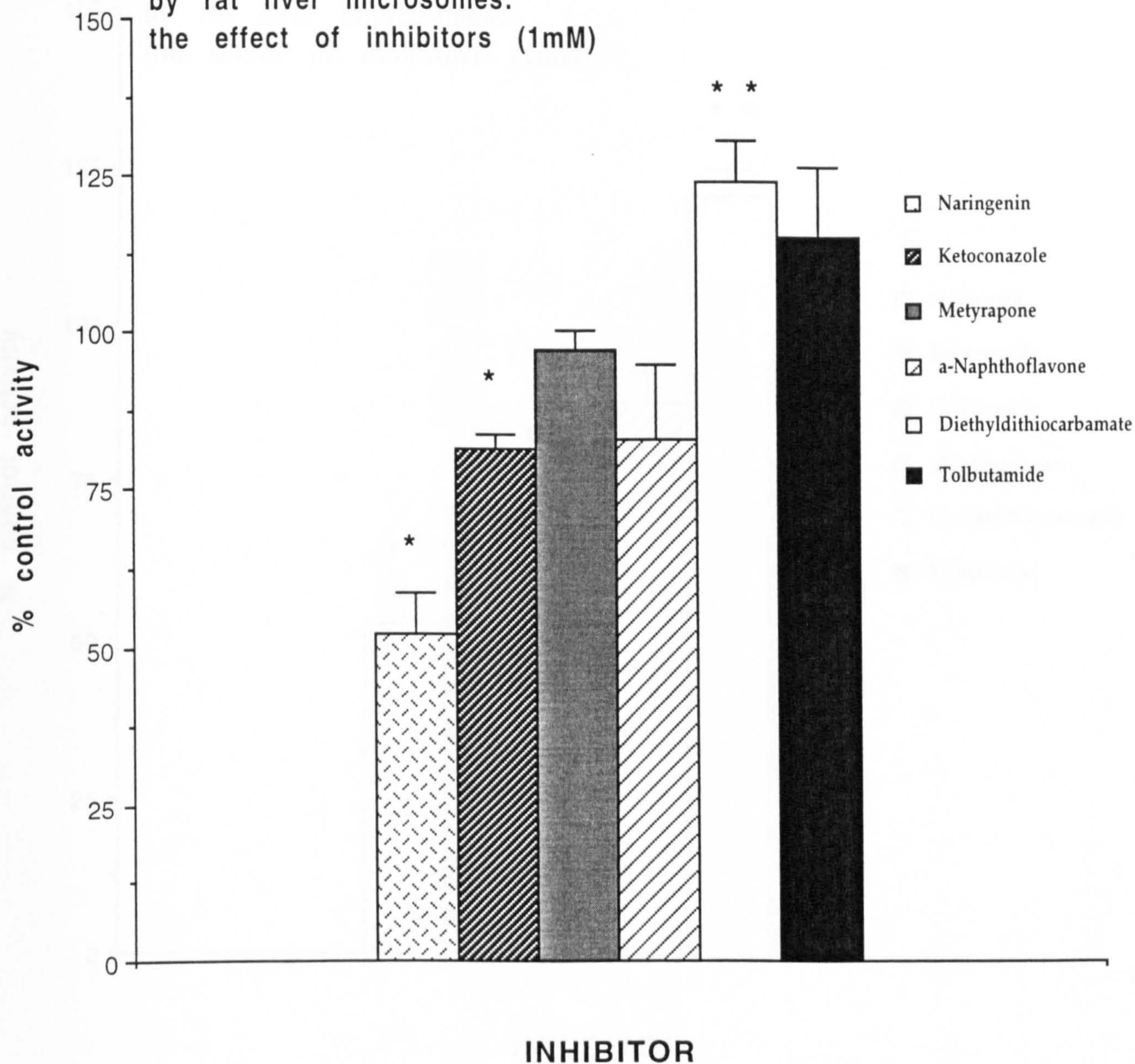
Individual values are given in appendix I

Further studies with parathion (200 μ M) and inhibitors (100 μ M) showed that the CYP3A inhibitors, with the exception of troleandomycin, produced the greatest decrease in activity (figures 10.5 and 10.6). Naringenin had the greatest effect (approximately 40% inhibition) on formation of both paraoxon and *p*-nitrophenol from parathion while quercetin and ketoconazole inhibited formation of both metabolites by about 25%. The other compounds had little effect on formation of *p*-nitrophenol or paraoxon and they appeared to be affected in parallel by the inhibitors.

Studies with parathion (20 μ M) and inhibitors (100 μ M) showed that troleandomycin slightly increased metabolite formation, whereas naringenin had the greatest inhibitory effect (approximately 50% inhibition) on formation of both paraoxon and *p*-nitrophenol (figures 10.7 and 10.8). The profile of inhibition with quercetin and ketoconazole (approximately 25% inhibition) at this parathion concentration was similar to that observed with the 200 μ M substrate concentration. Again the other compounds had little effect on formation of *p*-nitrophenol or paraoxon.

Sulphaphenazole had no inhibitory effect on paraoxon or *p*-nitrophenol formation from parathion (5 μ M and 200 μ M), in fact it produced slight increases in activity compared to control. Paraoxon and *p*-nitrophenol formation from 5 μ M parathion was increased by 7.7% \pm 5.9% and 32% \pm 18.5%, respectively. Levels were increased by 21% \pm 12.4% and 16.0% \pm 18.2%, respectively, from 200 μ M parathion.

Figure 10.3
Production of paraoxon from parathion (200 μ M)
by rat liver microsomes:
the effect of inhibitors (1mM)



* $p < 0.05$, lower compared to control

** $p < 0.05$, higher compared to control

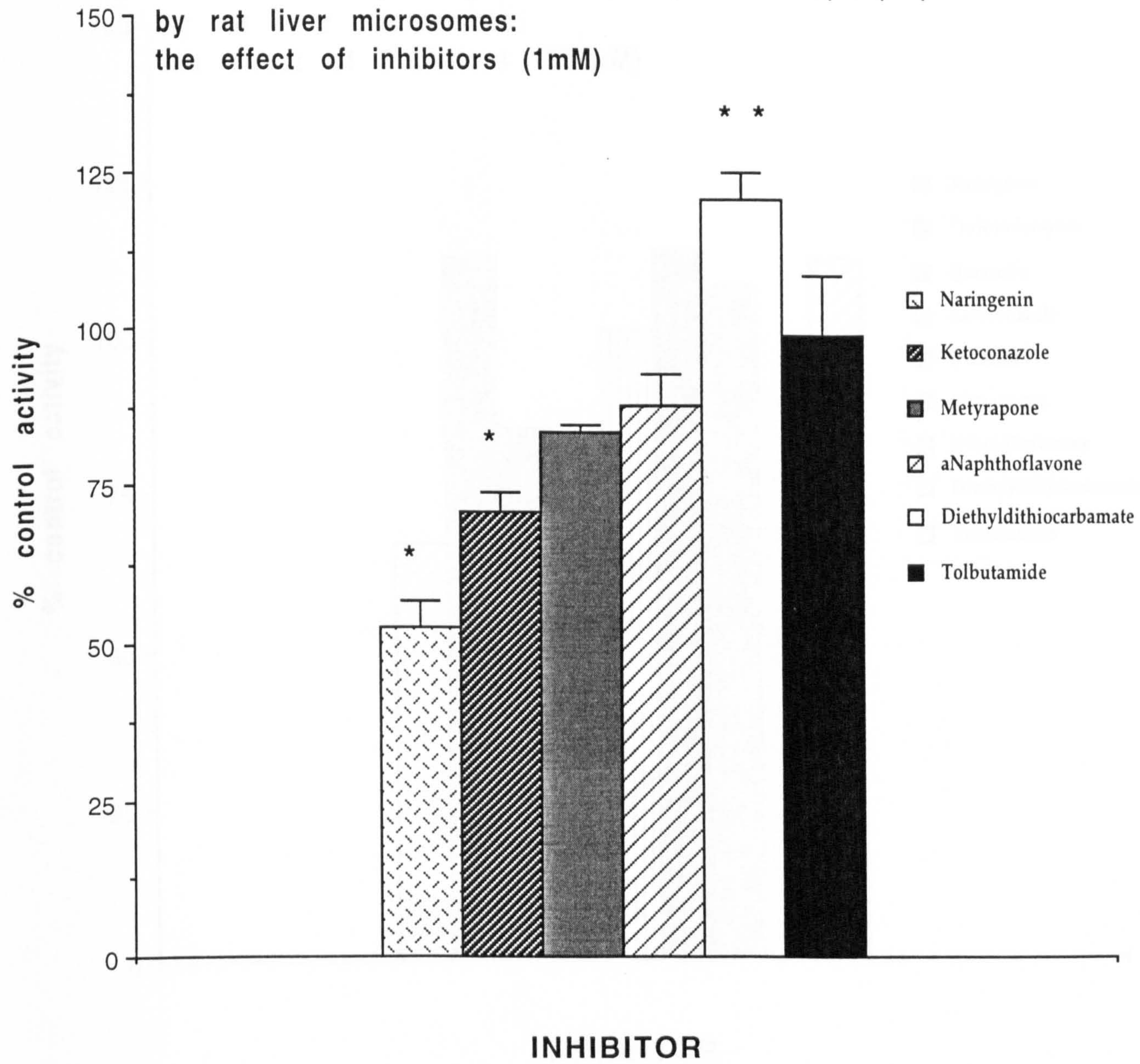
(ANOVA with modified t test)

Values are the mean \pm sem of $n=3$ rat liver microsomes

(duplicate determinations)

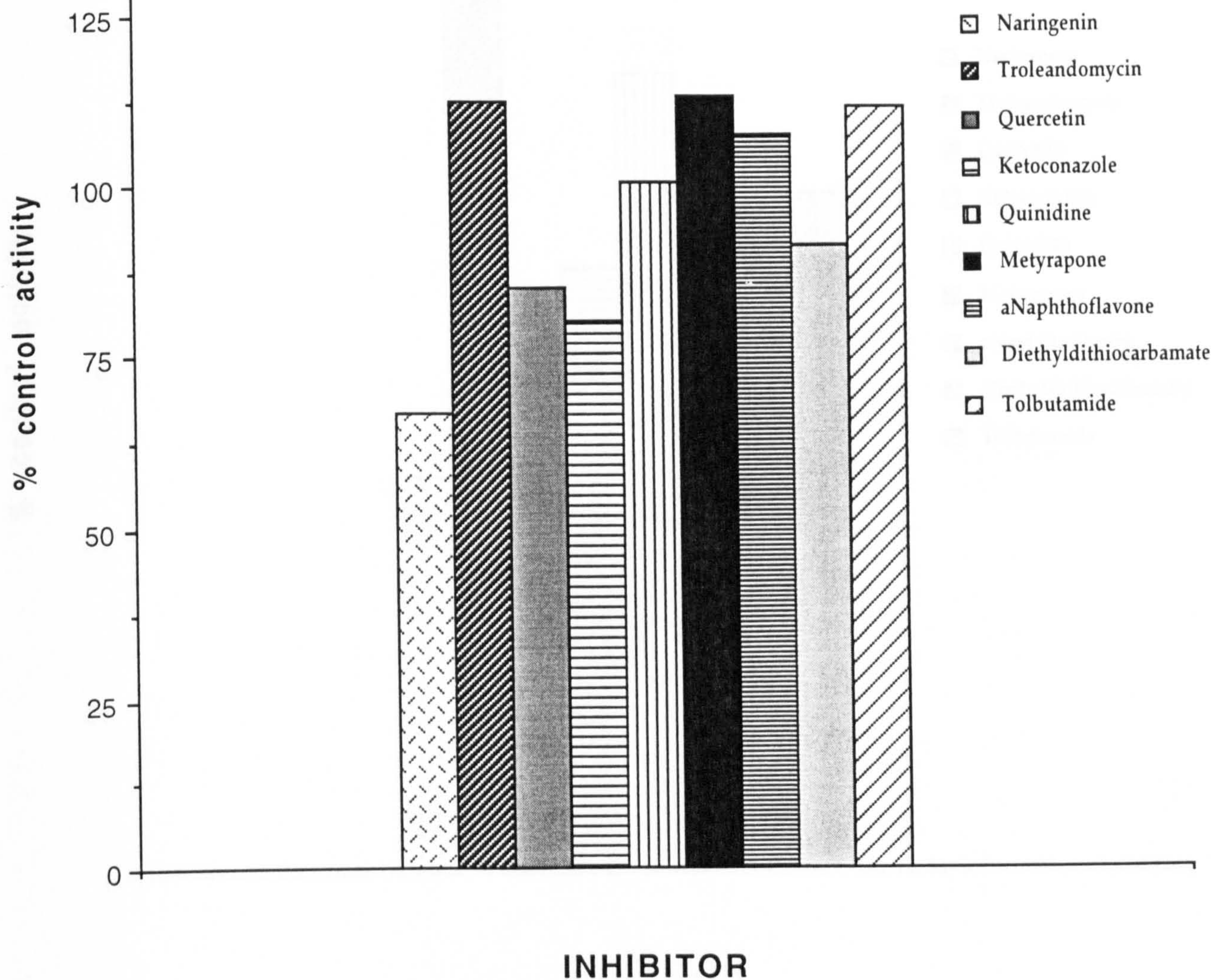
Individual values are given in appendix I

Figure 10.4
Production of p-nitrophenol from parathion (200μM)
by rat liver microsomes:
the effect of inhibitors (1mM)



* $p < 0.05$, lower compared to control
** $p < 0.05$, higher compared to control
(ANOVA with modified t test)

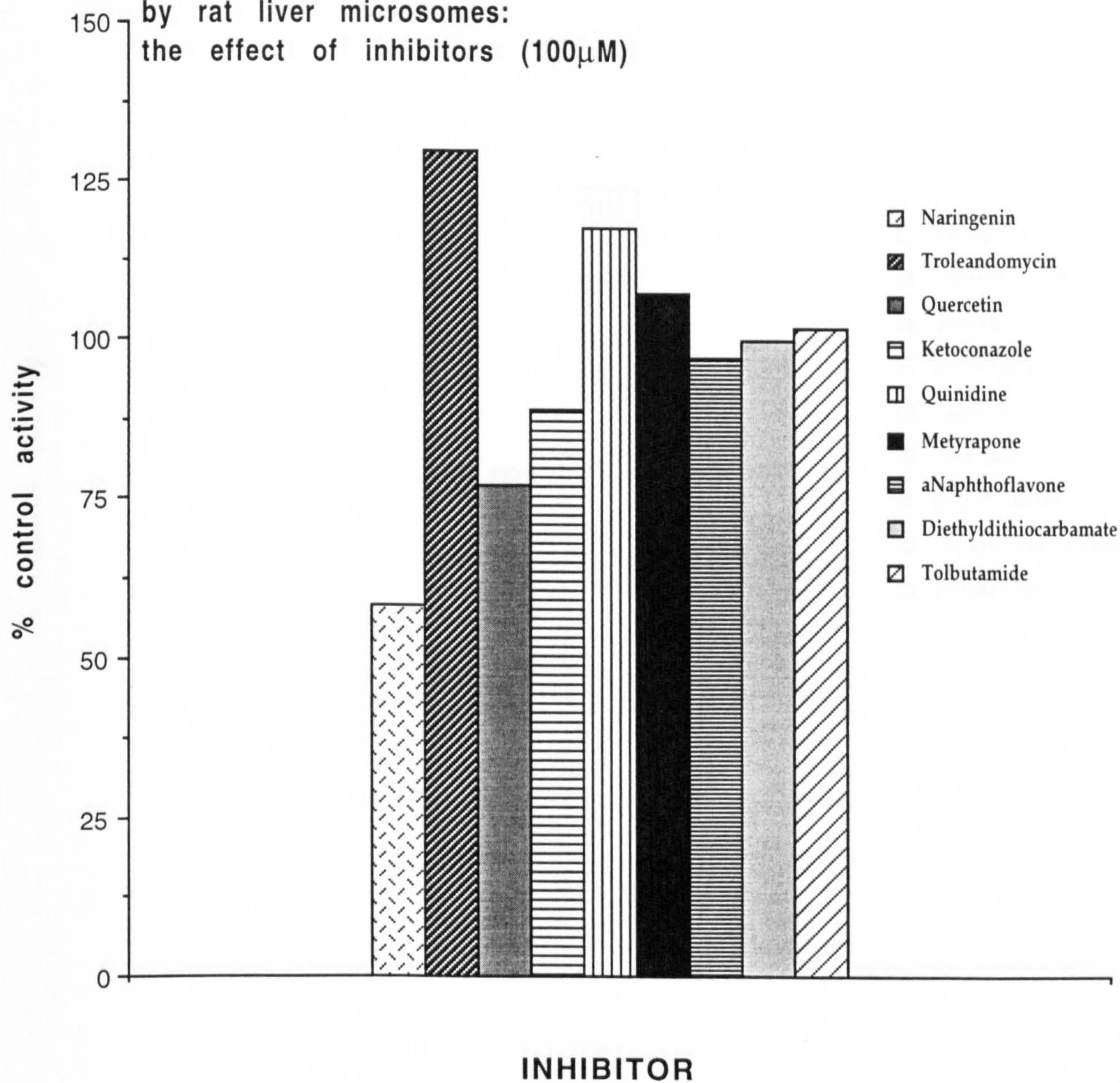
Figure 10.5
Production of paraoxon from parathion (200 μ M)
by rat liver microsomes:
the effect of inhibitors (100 μ M)



Values are the mean of 2 rat liver microsomal incubations
Individual data are given in appendix I

Figure 10.6

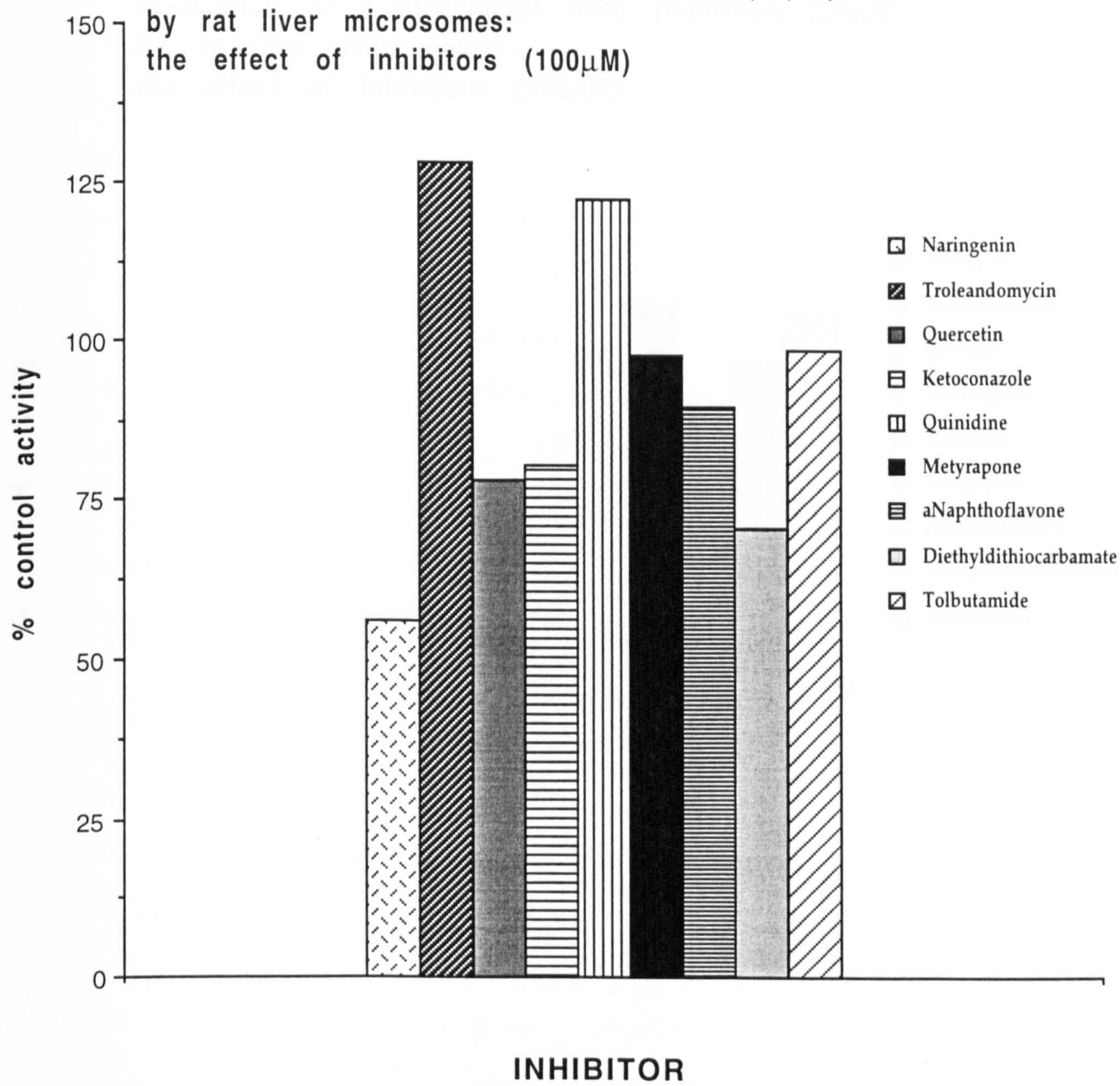
Production of p-nitrophenol from parathion (200 μ M)
by rat liver microsomes:
the effect of inhibitors (100 μ M)



Values are the mean of 2 rat liver microsomal incubations

Individual data are given in appendix I

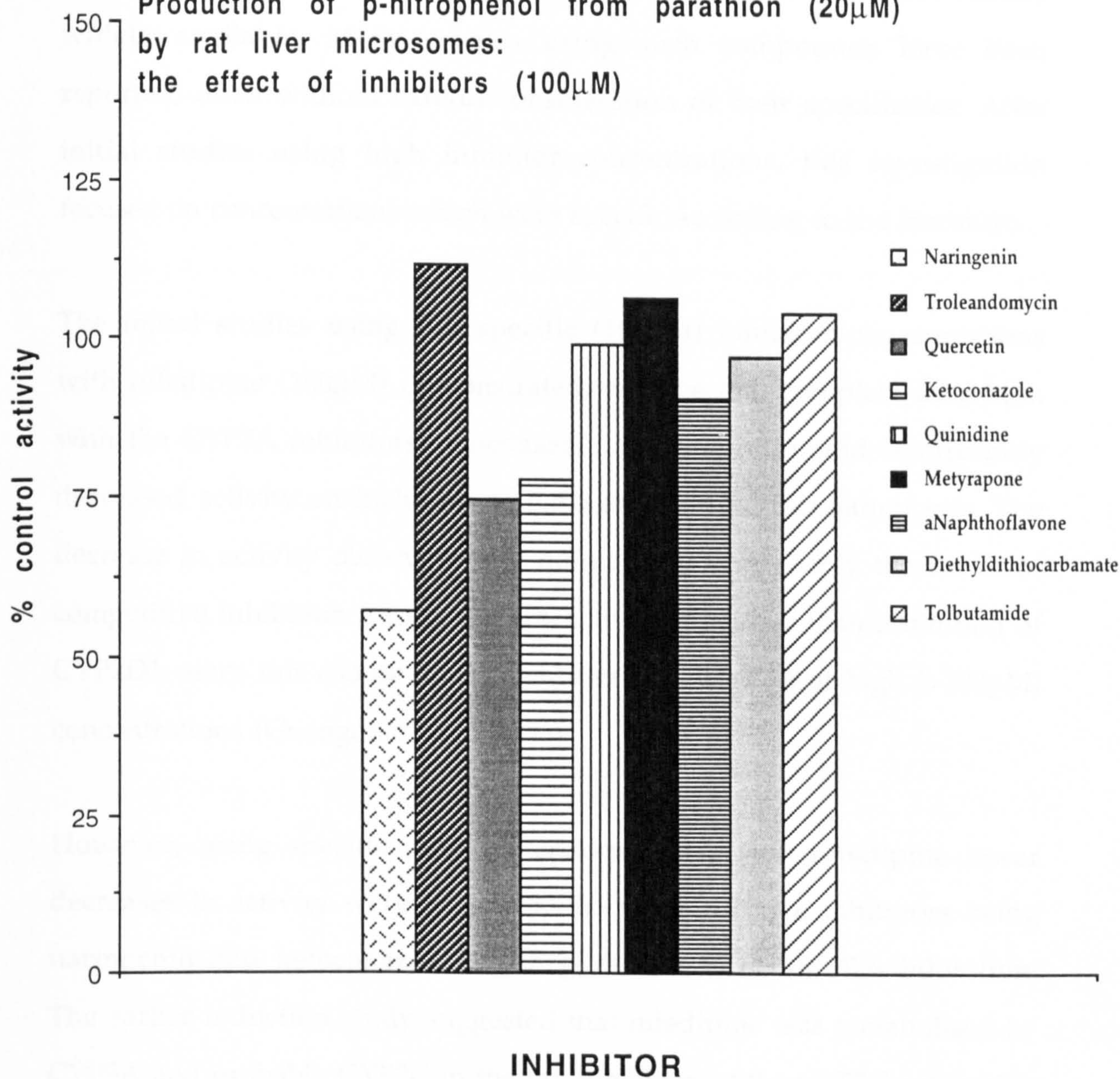
Figure 10.7
 Production of paraoxon from parathion (20 μ M)
 by rat liver microsomes:
 the effect of inhibitors (100 μ M)



Values are the mean of 2 rat liver microsomal incubations
 Individual data are given in appendix I

Figure 10.8

Production of p-nitrophenol from parathion (20 μ M)
by rat liver microsomes:
the effect of inhibitors (100 μ M)



Values are the mean of 2 rat liver microsomal incubations

Individual data are given in appendix I

For many years chemical inhibitors have been used as convenient tools with which to study metabolism *in vitro*, mainly because they are usually widely available. Many studies using such compounds have been reported, often without careful consideration of their specificities. After initial studies using high inhibitor concentrations, this investigation focused on concentrations which were specific according to the literature.

The initial studies using non-specific (100 μ M) inhibitor concentrations with nifedipine (100 μ M) demonstrated, as expected, complete inhibition with the CYP3A inhibitors ketoconazole and quercetin, and significantly decreased activity on inclusion of naringenin in the incubation mix. The decrease in activity observed with quinidine was probably the result of competitive inhibition for the CYP3A active site, rather than inhibition of CYP2D1, since this compound is a substrate for CYP3A at high (>100 μ M) concentrations (Guengerich *et al*, 1986).

However, using specific inhibitor concentrations and nifedipine lesser decreases in activity were observed, the most effective inhibitors being naringenin and ketoconazole which gave rise to about 70% inhibition. The earlier induction study suggested that nifedipine was metabolised by CYP3A and probably CYP2C in the rat. Inhibitors of the CYP2C subfamily, such as sulphaphenazole which inhibits human CYP2C9, were not investigated, but it is possible that inclusion of both CYP3A and CYP2C inhibitors (or antibodies) in the incubation would have completely inhibited the reaction.

Literature reports are conflicting regarding the specificity of troleandomycin as an inhibitor of CYP3A reactions. For example, a study by Chang and co-workers (1994) using HepG2 hepatoma cells expressed with human cytochromes P450, demonstrated that a 20 μ M concentration of troleandomycin yielded maximal complex formed with P450 haemoproteins. Conversely, others (Ono *et al*, 1996) using an *in vitro* system similar to Chang *et al* (and also human liver microsomes), reported that 1mM troleandomycin was necessary to inhibit CYP3A-mediated reactions. Troleandomycin appears to be very slow-acting in its role as a suicide inhibitor, with some researchers (Chang *et al*, 1994) reporting the necessity for pre-incubation times of thirty minutes or more. These experiments used a pre-incubation time of ten minutes which may have contributed to the lack of observed parathion inhibition.

The CYP3A inhibitors appeared to have the greatest effect on parathion metabolism and, although they never produced complete inhibition, none of the other compounds were remarkable. Even CYP1A, which was implicated in parathion metabolism by the induction study, did not appear to be involved in the reaction in control rat microsomes since minimal inhibition was observed with α -naphthoflavone. However, it should be noted that constitutive levels of CYP3A2 and CYP1A2 are only 17% and 2% of the total P450 pool, respectively, (Nedelcheva and Gut, 1994) and therefore maximal decreases in activity would not be possible with individual inhibitors unless parathion metabolism was exclusive to one of these isoforms.

Further experiments with sulphaphenazole concentrated on investigating a role for CYP2C11 in parathion metabolism as this the major constitutive rat isoform. It has been demonstrated that CYP2C11 has 1.20 nmoles P450/

mg microsomal protein, and accounts for 54% of the total P450 pool (Nedelcheva and Gut, 1994), compared to 0.39 nmoles P450/ mg microsomal protein for CYP3A2 (Guengerich *et al*, 1982). Surprisingly, sulphaphenazole had no inhibitory effect on parathion metabolism at high (200 μ M) or low (5 μ M) substrate concentrations. Tolbutamide was also an ineffective inhibitor of the reaction. Recent studies (Eagling *et al* 1998) comparing the selectivity of P450 inhibitors in human and rat liver microsomes appear to help elucidate the problem. Eagling *et al* demonstrated that sulphaphenazole was a selective inhibitor of tolbutamide hydroxylation in human liver microsomes but failed to inhibit the reaction in rat liver microsomes. Moreover, it has been suggested (Boobis *et al*, 1990) that competitive inhibitors (such as tolbutamide and sulphaphenazole) do not act via direct competition at the active site, and that the inhibitory site is different between species.

In summary, CYP3A was shown to participate in parathion metabolism in control rat microsomes, but it was not the only P450 isoform involved. It is possible that CYP2C played a role in the reaction but the present data did not support this hypothesis. *p*-Nitrophenol and paraoxon formation appeared to be affected in parallel by the inhibitors which indicated that the same P450 isoform(s) may be involved in both the activation and detoxification of parathion. These studies also suggested that nifedipine, although used by many researchers (Niwa *et al*, 1995; Shimada *et al*, 1997) as a good marker substrate for rodent CYP3A activity, may not be specific for this cytochrome P450 subfamily in the rat.

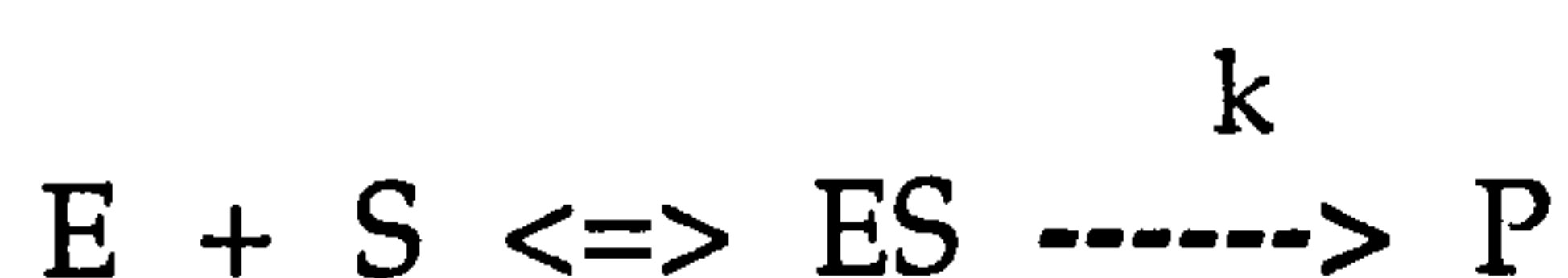
Chapter 11

Chapter 11 The kinetics of parathion metabolism

11.1 Introduction

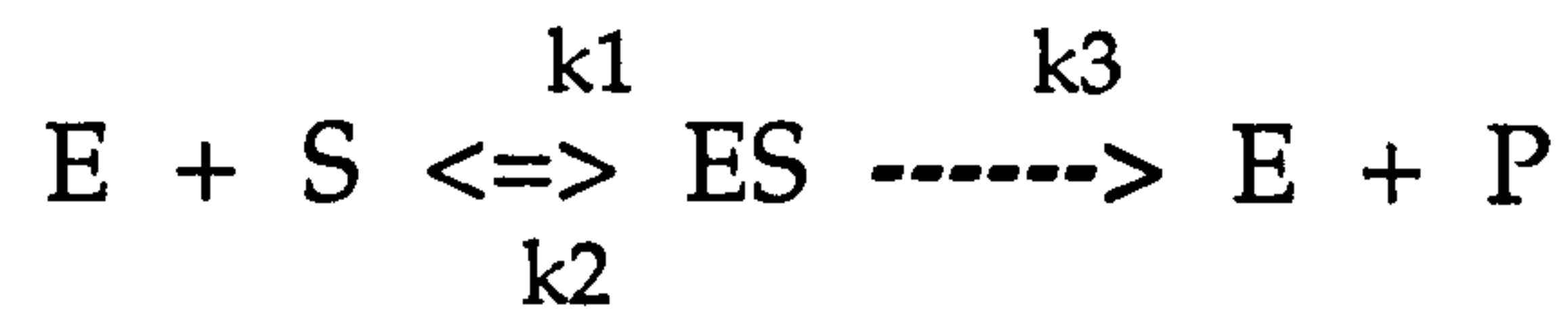
It was recognised that enzyme activities measured *in vitro* using routine high substrate concentrations may not accurately reflect activities *in vivo*. Furthermore, high substrate concentrations may not be physiologically important, particularly in the case of highly toxic phosphorothioates. Therefore a kinetic analysis of parathion metabolism was undertaken on the premise that this should yield a better assessment of the *in vivo* significance of these reactions. Further studies investigated the kinetics of parathion metabolism in the presence of a concentration of ketoconazole which should specifically inhibit CYP3A in order to indicate involvement of this isoform at substrate concentrations closer to those potentially encountered *in vivo*.

For most enzyme catalysed reactions, the rate of catalysis (v) varies with substrate concentration (s) in a saturable manner such that a plot of v against s shows a hyperbolic curve. At a fixed concentration of enzyme, v is linearly proportional to s when s is small, i.e. first order reaction kinetics. At high s , v is independent of s , i.e. zero order reaction kinetics. In 1913 Michaelis and Menten proposed a simple model to account for these kinetic characteristics. Thus:



where E is the enzyme, S is the substrate and ES is the enzyme-substrate complex and P is the product. The model assumes that the $E + S = ES$

step is in equilibrium. Briggs and Haldane (1925) realised that this may not always be the case and suggested the following adaptation of the Michaelis-Menten model:



where substrate binding to the enzyme is reversible, but not necessarily in equilibrium. This development enabled the equation known as the Michaelis-Menten equation to be formulated to describe the kinetics of enzyme catalysed reactions:

$$v = \frac{V_{\max} \cdot s}{K_m + s}$$

where v is the reaction rate, s is the substrate concentration, V_{\max} is the maximal reaction rate at enzyme saturation and K_m is the Michaelis constant.

K_m values range widely, for example the K_m for CO_2 binding to carbonic anhydrase is 8mM, while the K_m for ATP binding to pyruvate carboxylase is 60 μ M. The K_m value for an enzyme has two applications, firstly it is the concentration of substrate at which half the active sites are filled. Secondly, it reflects the affinity of an enzyme for a substrate, where a high K_m shows low affinity, and *vice versa*. The K_m is an intrinsic characteristic of enzyme-substrate binding and, within a given assay, is often referred to as the apparent K_m , which is unaffected by enzyme concentration or localisation.

The V_{\max} is a direct measure of the maximum rate of the catalysed reaction when all the enzyme's active sites are filled and, as such, is dependent on the amount of enzyme present in the tissue under investigation. For most physiological pathways, substrate concentrations *in vivo* are usually well below those needed for V_{\max} conditions. In the case of xenobiotic metabolism, however, it is possible for substrate concentrations to reach these levels. For example the metabolism of ethanol by alcohol dehydrogenase, or paracetamol conjugation with glucuronide in overdose. The V_{\max} can be used to determine the turn over rate of an enzyme if the enzyme concentration is known. The turnover number is equivalent to the rate constant k_3 in the above equation and is defined as the number of substrate molecules converted into product per unit time at fully saturable conditions. Like K_m , turn over numbers can vary widely, for example, carbonic anhydrase has a turnover number of $6 \times 10^5 \text{ s}^{-1}$ while glutathione-S-transferases have turnover numbers between 0.1 and 35 s^{-1} .

K_m and V_{\max} parameters may be derived by using non-linear regression to fit the Michaelis-Menten equation to the data set. The determination is done by adjustment of the values of V_{\max} and K_m for the known values of v and s until the sum of errors between the values of v and the calculated values of v is minimised. This is fairly easily achieved with the current widespread availability of computers.

Other methods for the calculation of K_m and V_{\max} rely on linear transformations of the Michaelis-Menten equation. The most popular is the double-reciprocal plot described by Lineweaver-Burke (1934). This plots $1/v$ against $1/s$, where the y-axis intercept is equivalent to $1/V_{\max}$ and the x-axis intercept is equal to $-1/K_m$. However, the plot distorts the

appearance of experimental error in the primary observations of v , especially at low substrate concentrations.

Other straight line transformations have been developed that eliminate the error of the double-reciprocal plot, but are still not entirely free of distortion. For example, a plot of v against v/s results in a straight line, where the slope represents $-K_m$ and the y-axis intercept is the V_{max} . This is known as the Eadie-Hofstee plot. The Eadie Hofstee plot is often used to illustrate the involvement of multiple enzymes in a metabolic process. A single enzyme would be expected to yield a straight line while multiple enzymes would give rise to an intersecting series of lines or a characteristic “hockey-stick” appearance.

11.2 Methods

Liver microsomes were prepared from 3 male Wistar rats weighing approximately 250g by the method described in chapter 7. The microsomal protein concentration of the preparations was determined by the method of Lowry adapted by Peterson (1977) using bovine serum albumin as standard, as described in chapter 7. Paraoxon and *p*-nitrophenol formation from parathion (5 μ M - 500 μ M) was determined for control (vehicle only) and ketoconazole (5 μ M) inhibited activities by the method described in chapter 8.

The kinetic parameters of parathion metabolism were investigated by fitting the data of paraoxon and *p*-nitrophenol formation verses substrate concentration for control and ketoconazole inhibited data using an iterative non-linear regression program using the built-in Solver function in Excel. The data points were not weighted. Eadie Hofstee plots were also

constructed and V_{\max} (y intercept) and apparent K_m (slope) determined. Data was fitted by least squares linear regression where appropriate.

$$\text{One enzyme model} \quad V = \frac{V_{\max} S}{K_m + S} \quad (1)$$

$$\text{Two enzyme model} \quad V = \frac{V_{\max(1)} S}{(K_{m(1)} + S)} + \frac{V_{\max(2)} S}{(K_{m(2)} + S)} \quad (2)$$

where $K_{m(1)}$, $V_{\max(1)}$ correspond to high affinity, low capacity (substrate) site and $K_{m(2)}$, $V_{\max(2)}$ correspond to low affinity, high capacity (substrate) site.

11.2.1 The effect of ketoconazole on parathion kinetics

Preliminary experiments

The rates of *p*-nitrophenol and paraoxon formation had been shown to be linear with time and protein concentration to 10 minutes and 0.4mg protein per incubation, respectively, at saturating substrate concentrations. In order to define the kinetics of parathion metabolism, it was necessary to observe the rates of reaction over a 100-fold substrate range (5 μ M - 500 μ M).

Preliminary experiments determined conditions of linearity for the lowest substrate concentration (5 μ M) using the method described in chapter 8. Using this parathion concentration the rate of *p*-nitrophenol and paraoxon formation was linear with time and protein concentration to 2.5 minutes and 0.25mg protein per incubation, respectively (figures 11.1 and 11.2).

Figure 11.1

The formation of p-nitrophenol and paraoxon from parathion ($10\mu\text{M}$) by rat liver microsomes: the effect of varying time

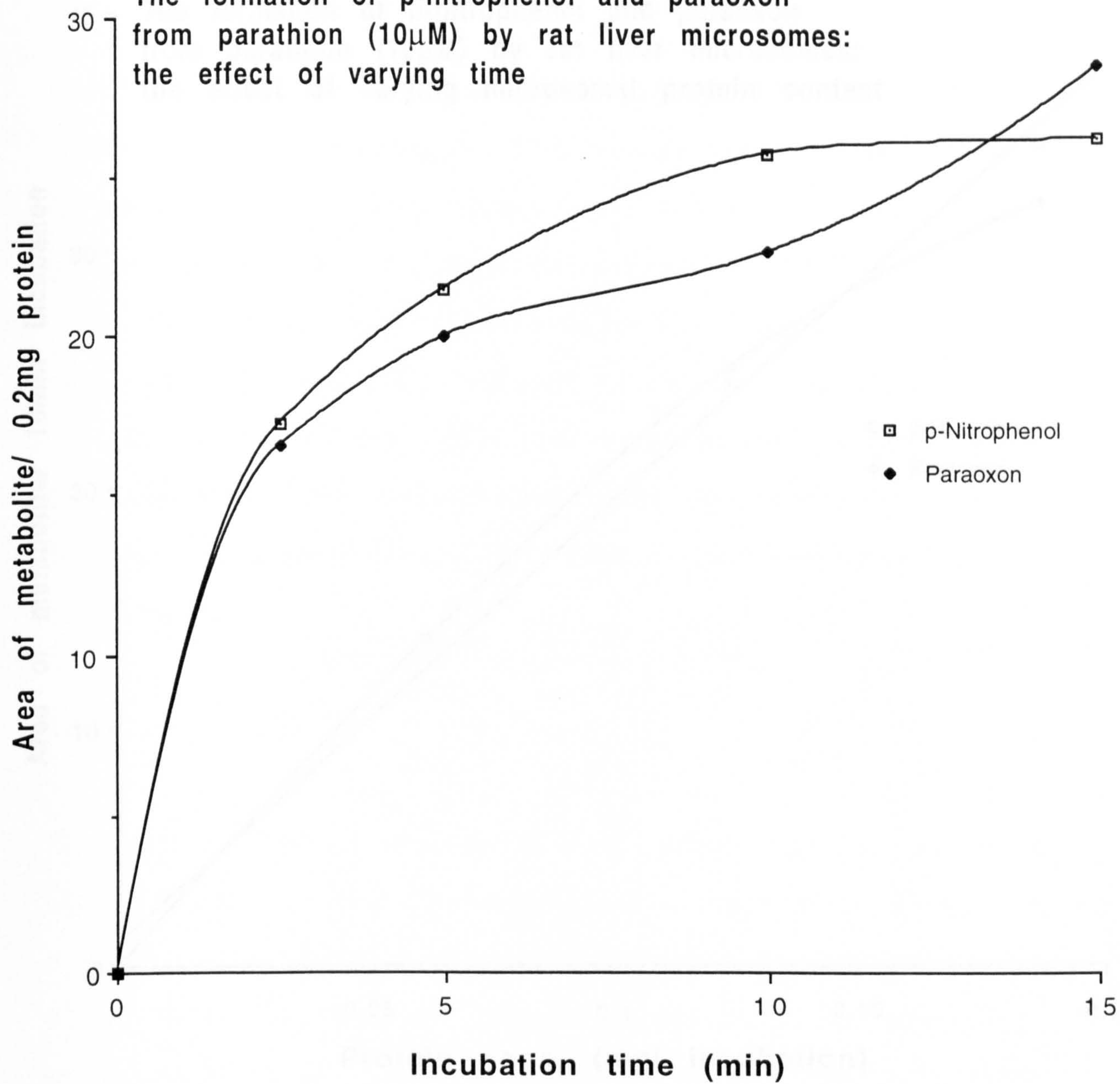
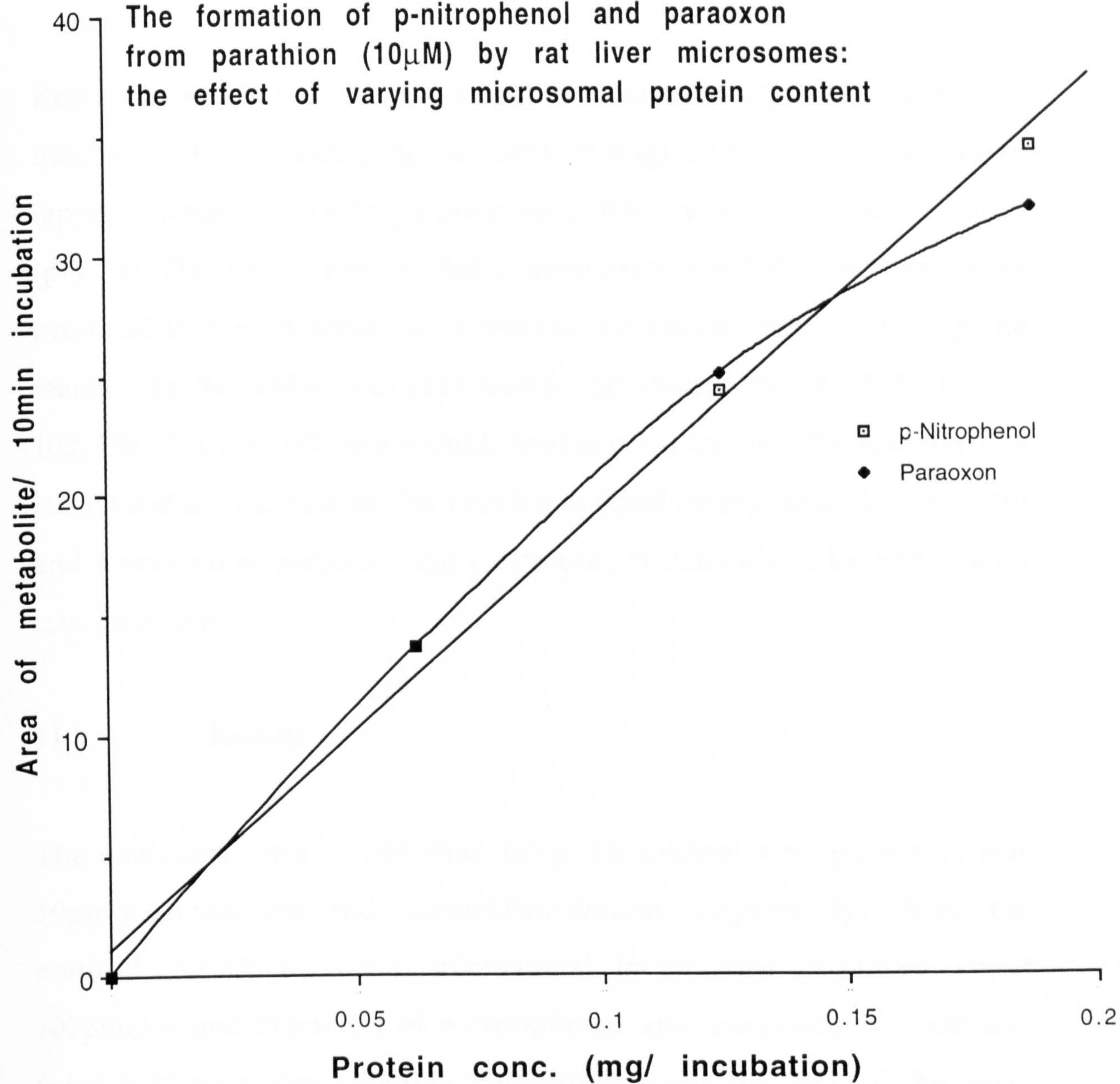


Figure 11.2

The formation of p-nitrophenol and paraoxon from parathion (10 μ M) by rat liver microsomes: the effect of varying microsomal protein content



Inhibitor

Ketoconazole was dissolved in methanol to give a 100mM stock solution. This was then diluted 1: 10 in methanol, followed by a further 1: 20 dilution to give a 0.5mM working stock.

Ketoconazole (5 μ l of 0.5mM stock) or methanol (5 μ l) was added to incubation tubes containing NADPH (0.3mg) and microsomal protein (approximately 0.25 - 0.3mg) made up to 0.5ml with Tris/ calcium buffer (pH 7.4). This gave a final inhibitor concentration of 5 μ M. The tubes were pre-incubated with inhibitor or vehicle for 10min prior to starting the reaction by the addition of parathion in 2 μ l aliquots (5, 10, 15, 25, 50, 75, 100, 150, 200, 300, 400 and 500 μ M, final concentrations). The reaction was terminated after 2.5min by the addition of 0.5ml perchloric acid (20%, w/v) and formation of paraoxon and *p*-nitrophenol determined by HPLC with u.v. detection.

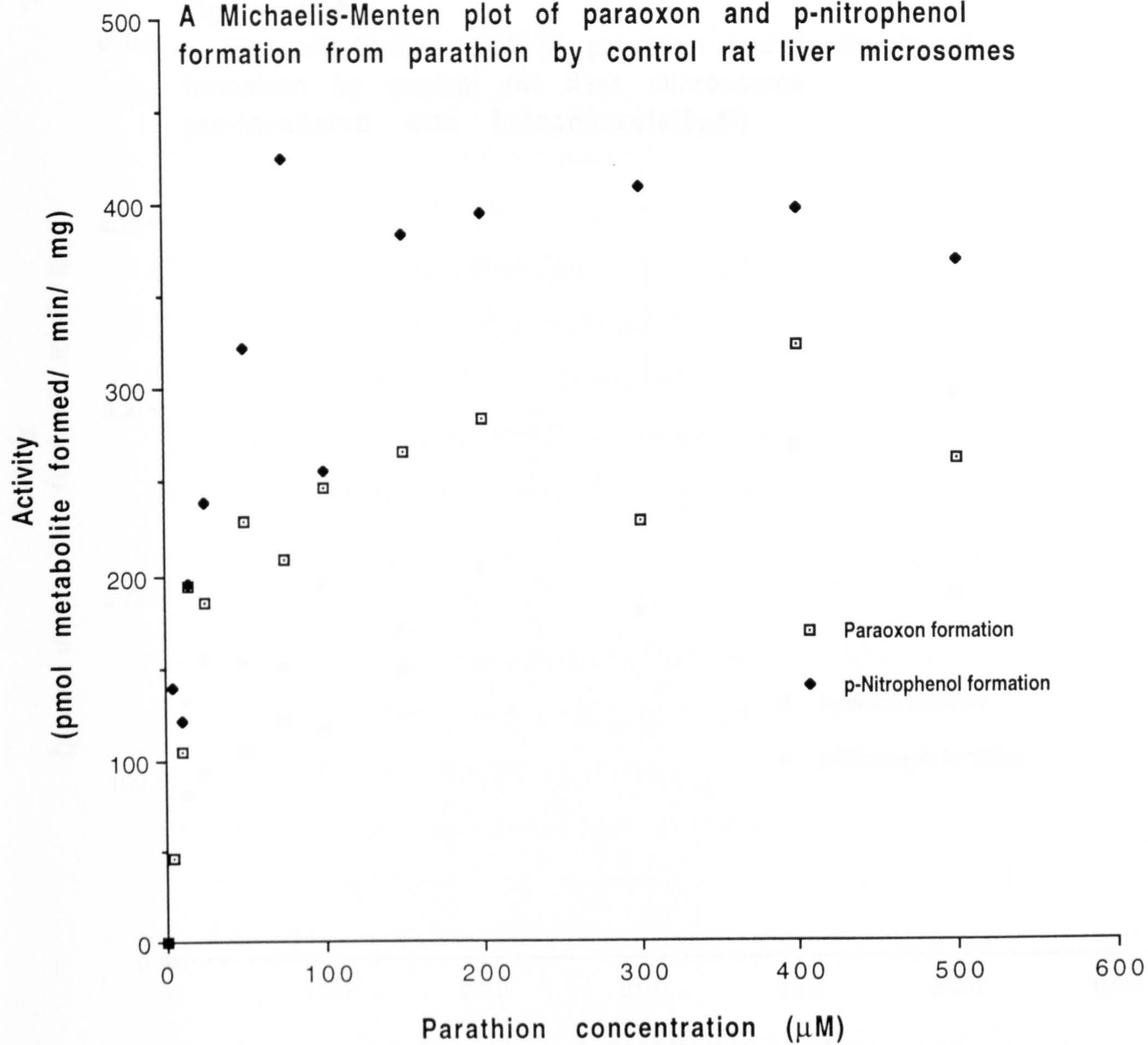
11.3 Results

The analytical limit of detection for *p*-nitrophenol and paraoxon was 10pmol/incubation and 35pmol/incubation, respectively. Using the method described above, microsomal incubations produced about 100pmoles and 90pmoles of *p*-nitrophenol and paraoxon, respectively, from 5 μ M parathion and this concentration was the limit of the assay sensitivity.

Control and ketoconazole inhibited plots of paraoxon and *p*-nitrophenol formation verses substrate concentration for one rat are shown in figures 11.3 and 11.4. Visual comparison of the inhibited plot with control suggested approximately 50% inhibition of paraoxon and *p*-nitrophenol

Figure 11.3

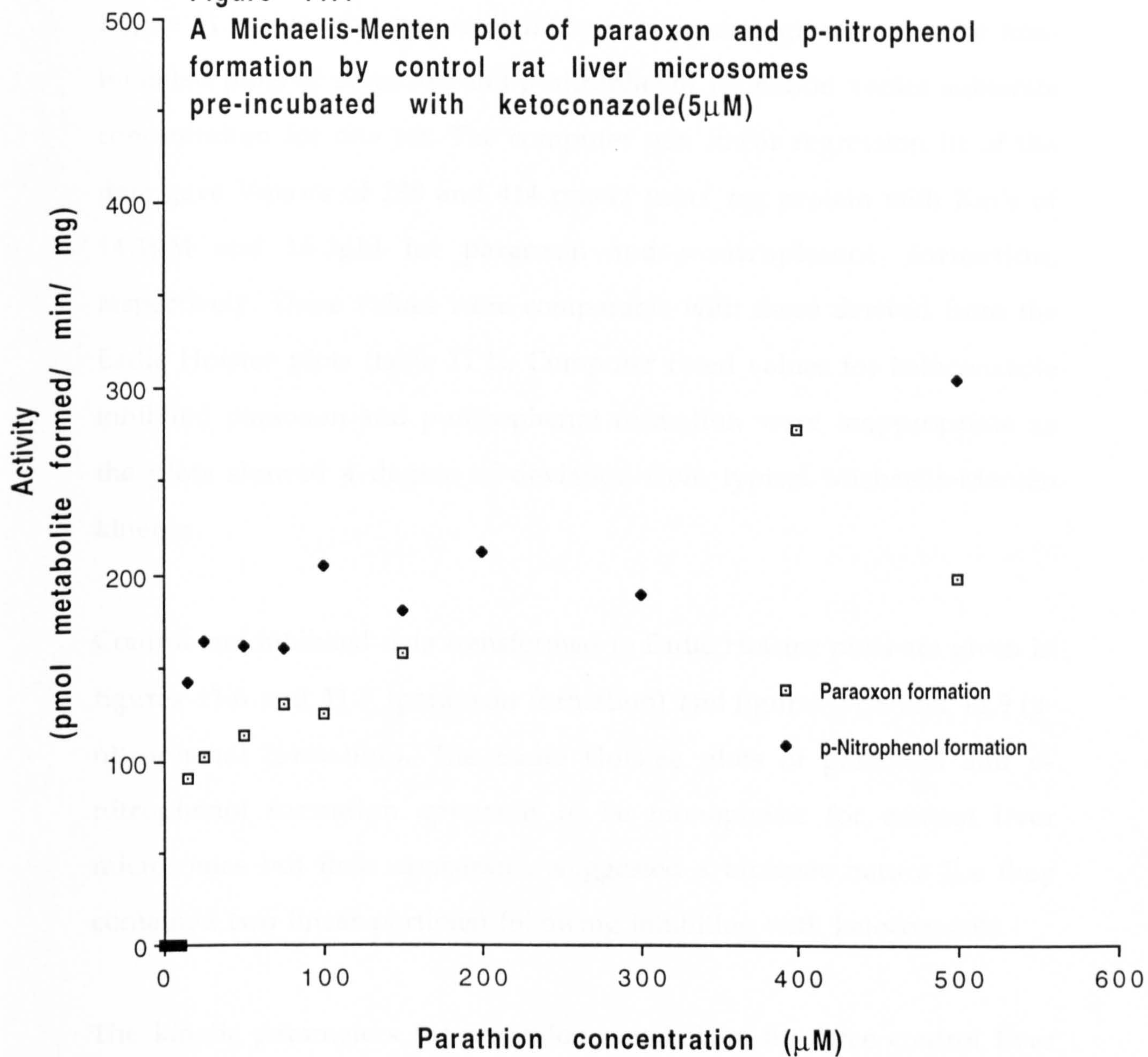
A Michaelis-Menten plot of paraoxon and p-nitrophenol formation from parathion by control rat liver microsomes



Values are the mean of duplicate determinations

Figure 11.4

A Michaelis-Menten plot of paraoxon and p-nitrophenol formation by control rat liver microsomes pre-incubated with ketoconazole(5 μ M)



Values are the mean of duplicate determinations

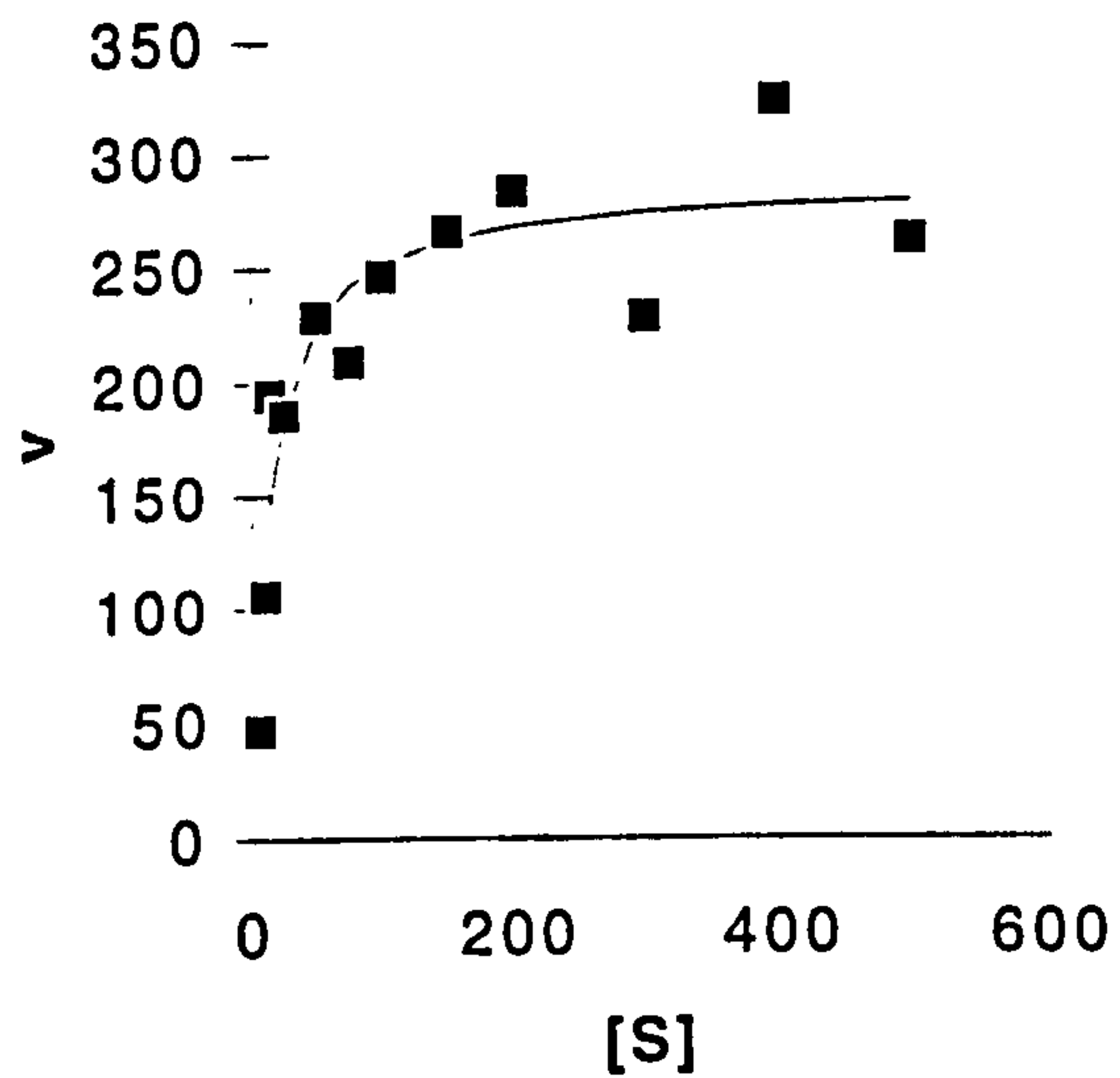
formation by ketoconazole at saturating substrate concentrations, ie those above 20 μ M. There was also complete inhibition of parathion metabolism at 5 μ M and 10 μ M substrate concentrations.

Figure 11.5 gives the computer fit by non linear regression for the non-inhibited plots of paraoxon and *p*-nitrophenol formation verses substrate concentration for one rat. The computer non linear regression fit of the data gave Vmax's of 288 and 414 pmol/ min/ mg protein with Km's of 14.1 μ M and 16.3 μ M for paraoxon and *p*-nitrophenol formation, respectively. These values were comparable with those derived from the Eadie Hofstee plots (table 11.1). Computer fitted values for ketoconazole inhibited paraoxon and *p*-nitrophenol formation were inappropriate as the plots showed a degree of deviation from typical Michaelis-Menten kinetics.

Control and inhibited data transformed to Eadie Hofstee plots are given in figures 11.6 and 11.7 (paraoxon formation) and figures 11.8 and 11.9 (*p*-nitrophenol formation). The Eadie Hofstee plots of paraoxon and *p*-nitrophenol formation appeared to be monophasic for control liver microsomes but their appearance suggested a biphasic nature (i.e they contained two linear portions) following inhibition with ketoconazole.

The kinetic parameters for parathion metabolism by three control liver microsomes were calculated from their Eadie Hofstee plots and are given in table 11.1. Similarly, the high and low substrate components of parathion metabolism kinetics following inhibition were calculated and are given in table 11.2.

Non Linear Regression

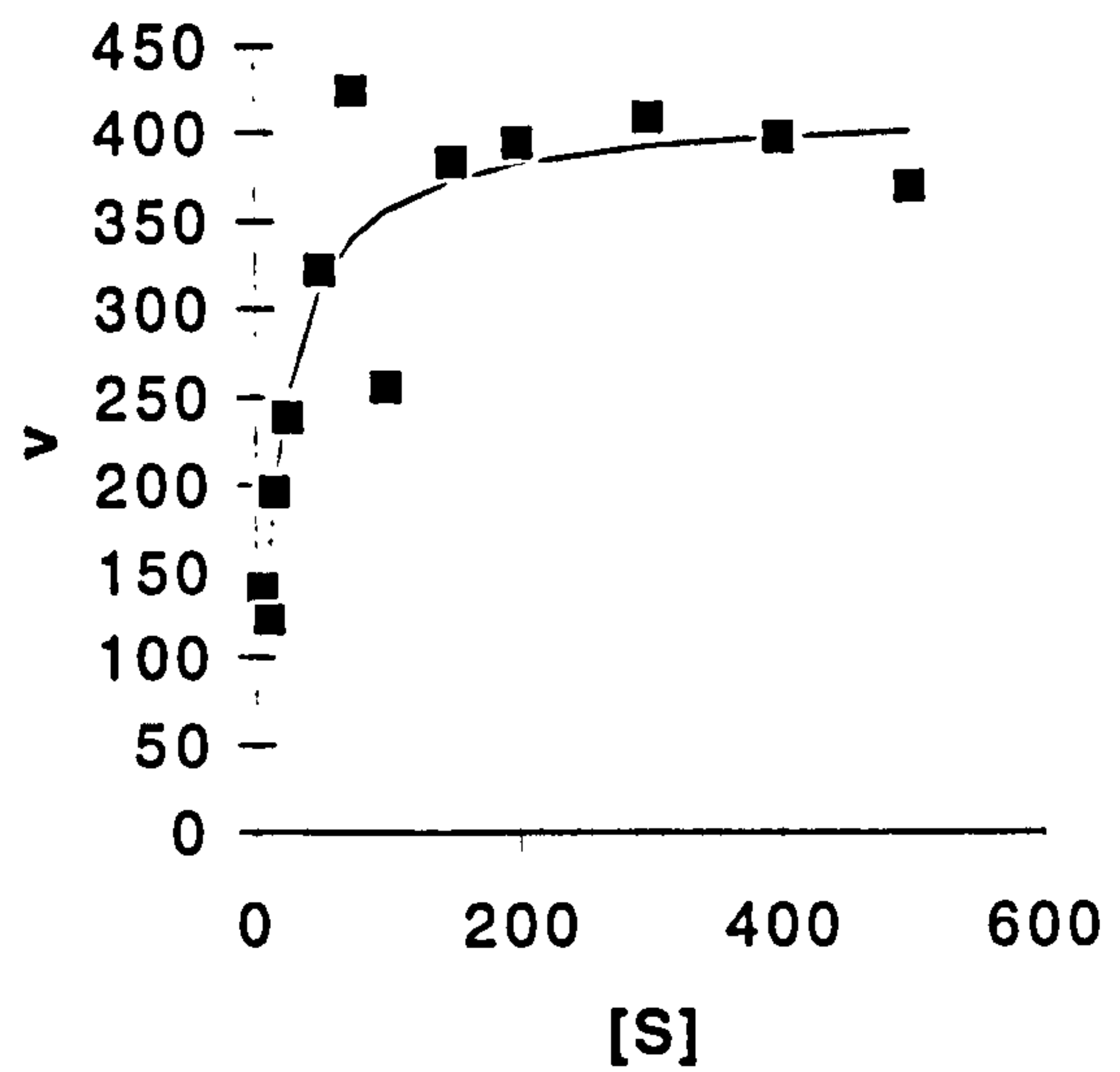


PARAOXON

Non Linear Regression

Km 14.105
Vmax 288

Non Linear Regression



p-NITROPHENOL

Non Linear Regression

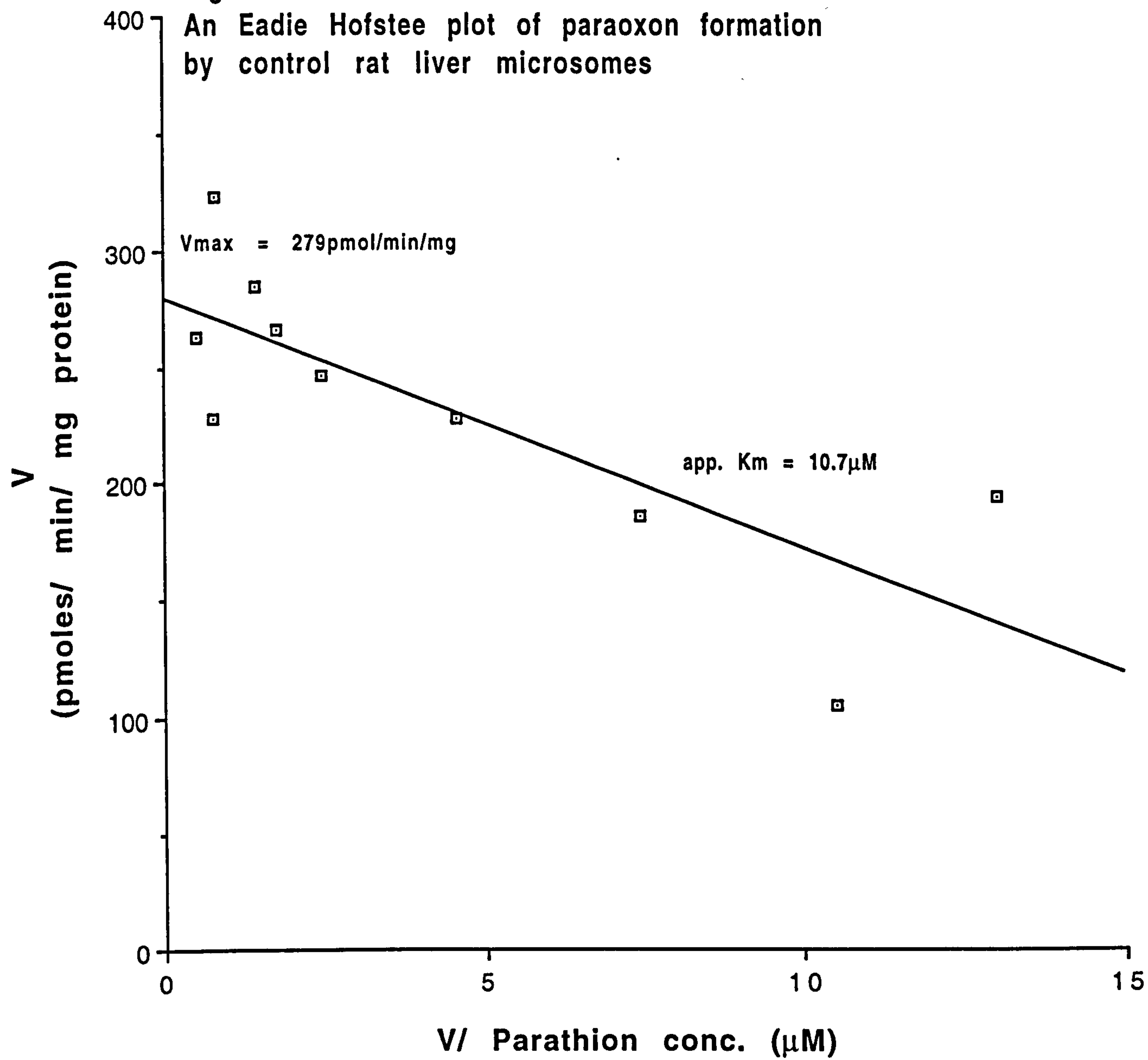
Km 16.339
Vmax 414

Figure 11.5

Non linear regression computer fit of paraoxon and *p*-nitrophenol formation by non-inhibited rat liver microsomes.

Figure 11.6

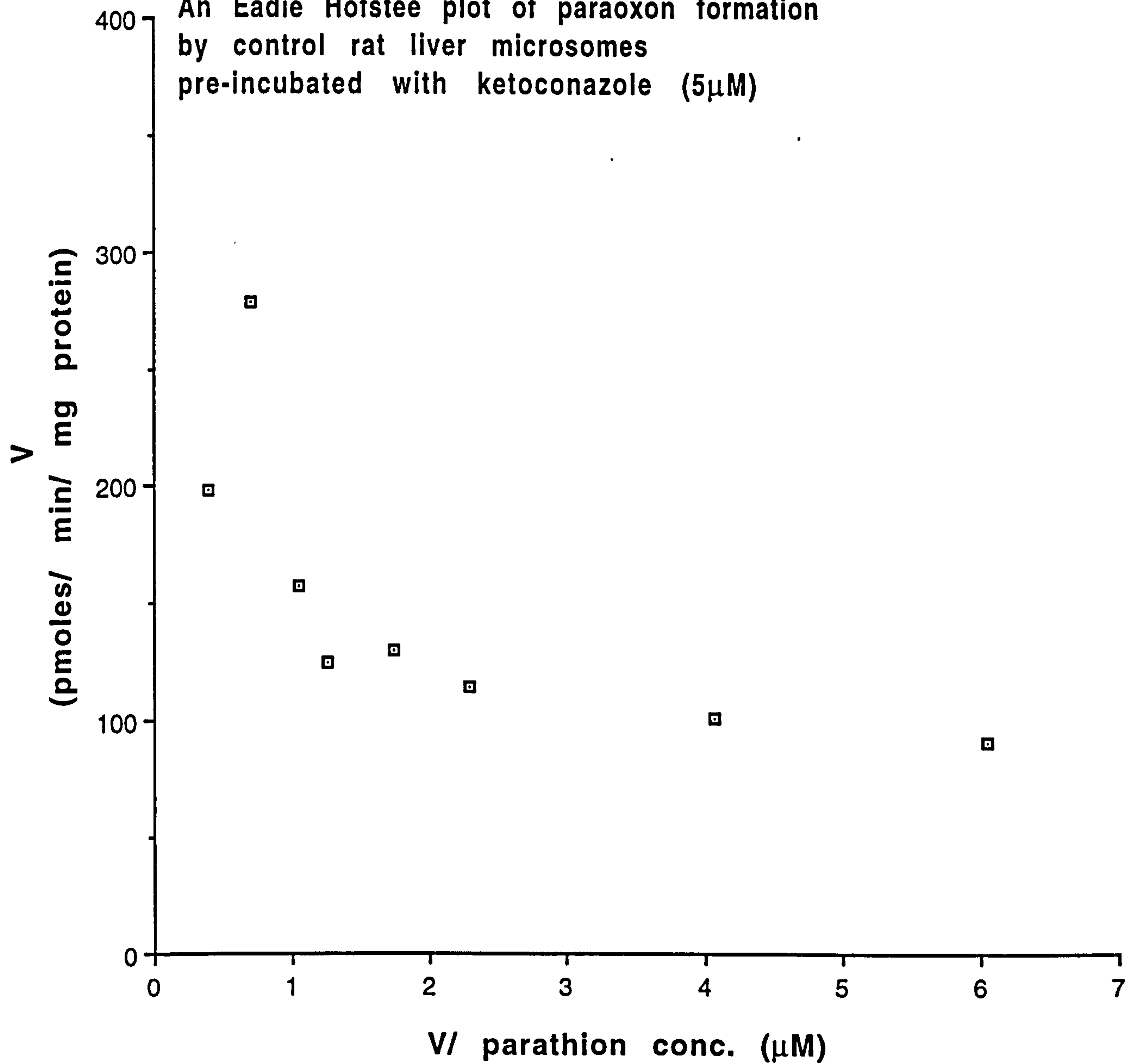
An Eadie Hofstee plot of paraoxon formation
by control rat liver microsomes



Values are the mean of duplicate determinations

Figure 11.7

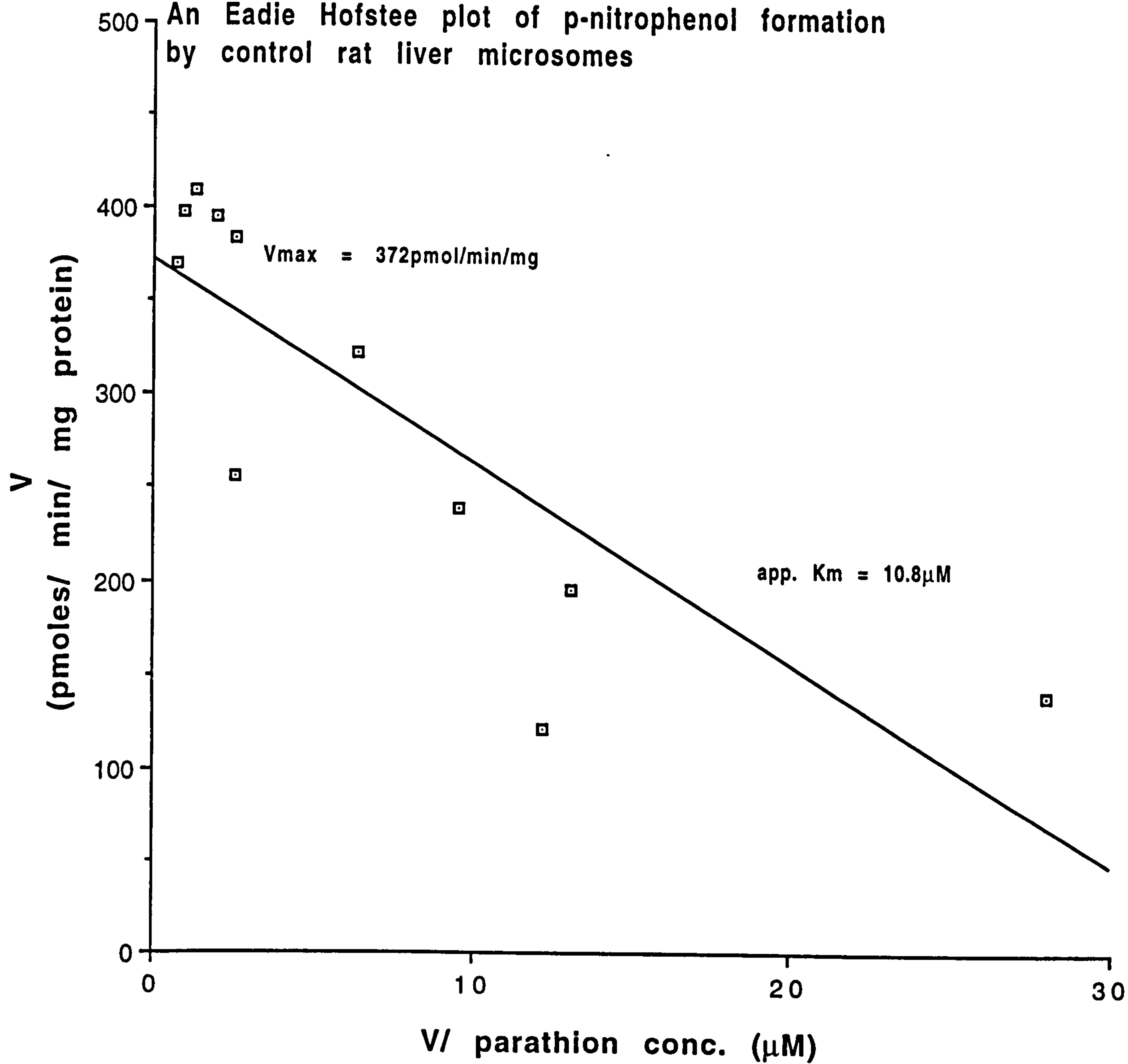
An Eadie Hofstee plot of paraoxon formation
by control rat liver microsomes
pre-incubated with ketoconazole (5 μ M)



Values are the mean of duplicate determinations

Figure 11.8

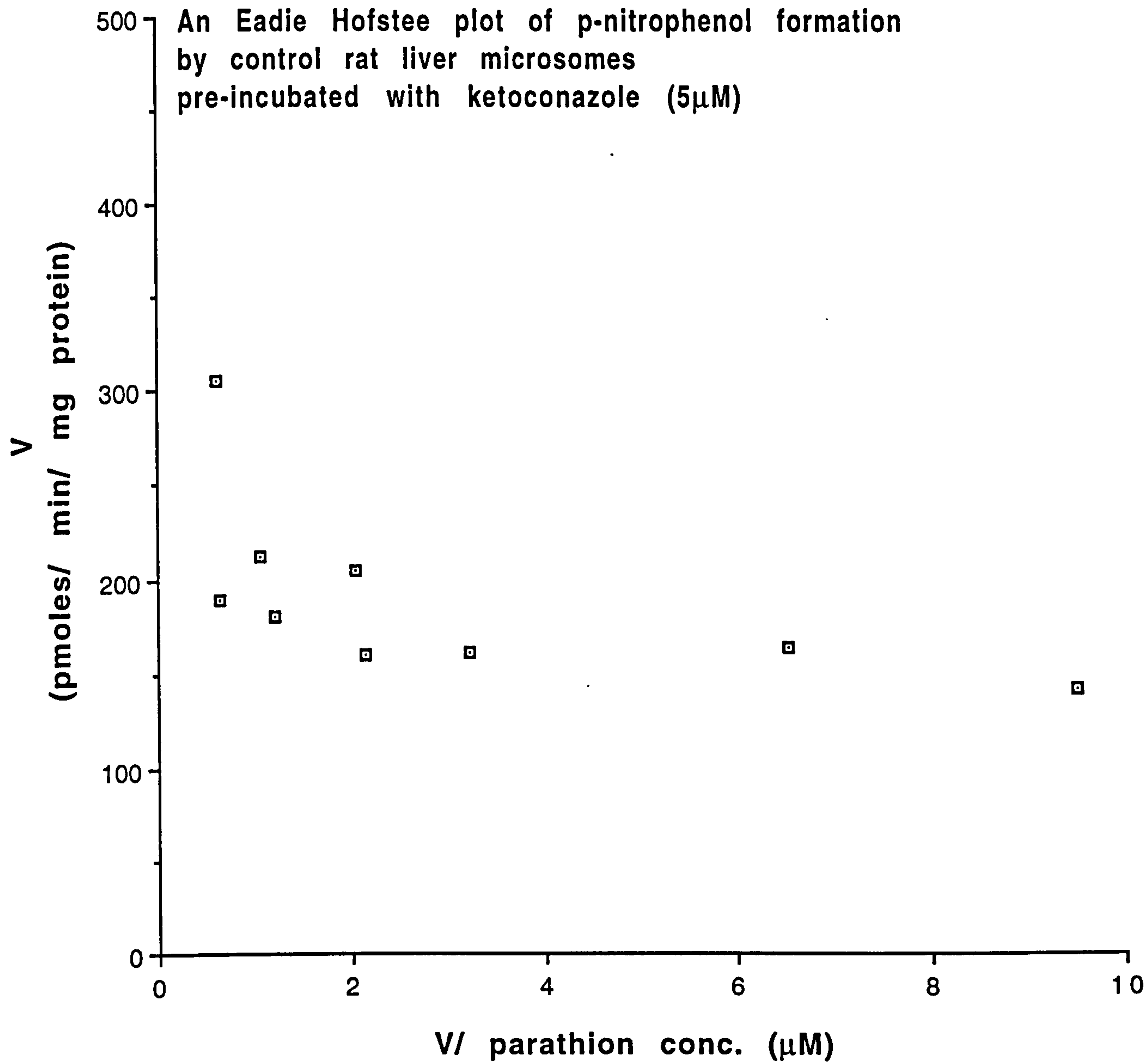
An Eadie Hofstee plot of p-nitrophenol formation by control rat liver microsomes



Values are the mean of duplicate determinations

Figure 11.9

An Eadie Hofstee plot of p-nitrophenol formation
by control rat liver microsomes
pre-incubated with ketoconazole (5 μ M)



Values are the mean of duplicate determinations

Table 11.1 The Vmax and apparent Km values for paraoxon and *p*-nitrophenol formation by control rat liver microsomes (n = 3). Values are derived from Eadie Hofstee plots and are the mean ± sem.

Metabolite	Vmax (pmol metabolite formed /min /mg protein)	apparent Km (µM)
Paraoxon	283.0 ± 2.1*	10.4 ± 0.25
<i>p</i> -Nitrophenol	349.7 ± 11.2	8.2 ± 1.4

* p<0.05, compared to formation of *p*-nitrophenol (paired t-test)

Table 11.2 The kinetic parameters for parathion metabolism by rat liver microsomes (n = 3) pre-treated with ketoconazole (5µM) *in vitro*. Values were derived from Eadie Hofstee plots and are the mean ± sem.

	Low substrate component		High substrate component	
Metabolite	Vmax (pmol/ min/ mg protein)	Km (µM)	Vmax (pmol/ min/ mg protein)	Km (µM)
Paraoxon	95.1 ± 12.5	42.8 ± 22.2	141.4 ± 18.2	14.8 ± 11.0
<i>p</i> -Nitrophenol	103.3@	21.4@	187.8 ± 13.7	2.04 ± 1.34

@n = 2 determinations

The Vmax for *p*-nitrophenol formation by control rat liver microsomes was higher than paraoxon, although their apparent Km’s were similar. Vmax’s determined for paraoxon and *p*-nitrophenol formation at the high substrate component were lower than control values, by 49.7% ± 6.4% and 46.3% ± 4.7%, respectively.

Early work by Norman *et al* (1974) using rat liver microsomes with [³⁵S] and [¹⁴C] labelled parathion at physiological pH showed that the amount of sulphur bound to microsomes in the absence of an NADPH-generating system was only approximately 2% of that bound in the presence of the generating system. Moreover, the V_{max} rate determined for sulphur binding was not statistically different from the V_{max} rate for paraoxon formation. The authors suggested that these observations provided strong evidence that the sulphur binding was a result of P450-mediated metabolism of parathion and that this correlated with paraoxon formation.

In the same publication Norman *et al* reported the rate of paraoxon formation by rat liver microsomes as 353 ± 26.7 pmol/ min/ mg protein and K_m $17.5\mu M \pm 4.3\mu M$, which was similar to the values (283 ± 2.1 pmol/ min/ mg protein and K_m $10.4\mu M \pm 0.25\mu M$) determined in this study. Likewise, early work by Nakatsugawa *et al* (1968) reported a K_m of $10.2\mu M$ for paraoxon formation from parathion by rat liver microsomes. However, Norman *et al* also demonstrated that as much as 20% of the paraoxon formed *in vitro* could be bound to the microsomes and therefore accurate measurements of paraoxon formation should include the concentration of free metabolite in the incubation media plus the amount bound to the microsomes.

In this study paraoxon standards prepared in the presence and absence of microsomal protein at the concentration used for incubations produced identical results inferring no loss of paraoxon by binding. Further

experiments using labelled parathion would be necessary to confirm this observation.

A recent study by Ma and Chambers (1995) of control rat liver microsomes showed that the V_{\max} for *p*-nitrophenol formation from parathion was 1.49 ± 0.15 nmol/ min/ mg and K_m $56.0\mu\text{M} \pm 18.8\mu\text{M}$. The kinetics for paraoxon formation were biphasic and gave K_m 's of $0.23\mu\text{M} \pm 0.10\mu\text{M}$ and $71.3\mu\text{M} \pm 16.4\mu\text{M}$ with V_{\max} 's of 3.62 ± 0.16 nmol/ min/ mg and 4.56 ± 1.38 nmol/ min/ mg, respectively. These researchers used a spectrophotometric technique to measure *p*-nitrophenol levels while production of paraoxon from parathion was measured indirectly by monitoring the amount of inhibition produced in an exogenous source of acetylcholinesterase (bovine brain) by the oxon generated. The different analytical techniques used in the Ma and Chambers investigation compared to this study may have contributed to the difference in kinetic values.

The physiological importance of the K_m 's determined in this study for paraoxon and *p*-nitrophenol formation is difficult to assess without knowledge of the concentration of toxin at the enzyme site/ receptor *in vivo*. Under conditions of equilibrium the parathion concentration at hepatic enzyme sites may approximate to the unbound plasma concentration. However, putative hepatic levels are likely to be considerably higher than in the plasma during the absorption phase. Estimates of toxin concentration at the enzyme site are further complicated by the fact that it has been shown that suspended rat hepatocytes absorbed parathion rapidly and reversibly until the intracellular concentration was >300-fold the ambient level (Nakatsugawa *et al*, 1980).

Sultatos *et al* (1985) has shown that the hepatic portal blood concentration of parathion peaked at $8.9\mu\text{M} \pm 1.9\mu\text{M}$ thirty minutes following an i.p. near lethal dose (13mg/ kg) to the mouse. In this extreme situation, and bearing in mind the potential for active hepatic uptake, parathion could be expected to be metabolised at V_{max} rates since the substrate concentration would be saturating.

The biphasic nature of the Eadie Hofstee plots following inhibition with ketoconazole suggested the presence of multiple forms of P450 which were capable of metabolising parathion to paraoxon and *p*-nitrophenol, but which were unequally sensitive to inhibition by ketoconazole. These data indicated that at least two enzymatic sites were involved, although allosteric changes of a single site could not be excluded.

Approximately 50% of the high substrate component for paraoxon and *p*-nitrophenol formation was not inhibited by the ketoconazole concentration used. This corroborates the data obtained for incubations with $20\mu\text{M}$ and $200\mu\text{M}$ parathion and ketoconazole in which approximately 25% inhibition was observed. Taken together these data suggested that about 25%-50% of parathion metabolism was probably mediated by CYP3A in control rat liver at saturating substrate concentrations. At least a proportion of the low substrate component(s) was not inhibited by ketoconazole which precludes involvement of CYP3A in this constituent of the reaction.

The K_m determined for *p*-nitrophenol formation at the high substrate component appeared to be decreased by ketoconazole, but the high variability of the values precluded any real conclusions for this observation. However, the decrease in K_m tends to corroborate the

deduction of others (Gibbs *et al*, 1997) that ketoconazole is a non-competitive inhibitor of CYP3A-mediated reactions. The observed decrease in V_{max} may therefore have resulted from a conformational change in the enzyme's binding site.

In summary, the kinetics of the reaction provided further evidence that several P450 isoforms are involved in parathion metabolism in control rat liver microsomes. It demonstrated that CYP3A was responsible for about 50% of parathion metabolism at saturating substrate concentrations and that isoform(s) other than CYP3A were involved at lower concentrations. Further studies, possibly including specific P450 antibodies, would be required to elucidate participation of individual P450 isoforms in parathion metabolism in the rat.

Chapter 12

Chapter 12 Parathion metabolism by human liver microsomes

12.1 Introduction

In this section of the thesis interindividual variations in capacity to metabolise parathion were determined in sixteen human liver microsomal preparations. Studies then concentrated on assessing the individual P450 isoform(s) involved in the reaction in man.

Many different strategies are currently employed in the identification of P450 isoforms responsible for the biotransformation of xenobiotics. These include the use of selective chemical inhibitors of P450 isoforms, commercially available human P450-expressed microsomes, and correlation analyses between the rates of metabolism of the xenobiotic and model P450 substrates. A combination of these approaches was used in the following chapters.

12.2 Methods

12.2.1 The subjects

Human livers (n = 16) were obtained from patients in Newcastle with approval of the Joint Ethical Committee of Newcastle and North Tyneside Health Authority and University of Newcastle, and from Professor G. Hawksworth, Aberdeen with approval of the Joint Ethical Committee of the Grampian Health Board. The livers were flash-frozen as soon as possible after removal and then stored at -70°C until use. Demographic details for the subjects are given in table 12.1.

Table 12.1 Demographic details for human liver samples

Lab. Number	Age	Sex	Smoking habits	Alcohol consumption	Drug history/ other details
M1 (HL12)	47	female	n.k.	n.k.	Liver function test normal. Subarachnoid haemorrhage
M2 (HL10)	63	female	n.k.	n.k.	Liver function test normal. Cerebral haemorrhage
M3 (22/9/93)	47	female	n.k.	n.k.	Liver function test normal. Subarachnoid haemorrhage
M4 (HL9)	55	male	n.k.	n.k.	Liver function test normal. Head injury
M5 (9/11/89)	45	female	n.k.	n.k.	Liver function test normal. Subarachnoid haemorrhage
M6 (HL15)	55	male	n.k.	n.k.	Liver function test normal. Head injury
M7 (HL14)	35	male	n.k.	n.k.	Liver function test normal. Subarachnoid haemorrhage
M8 (010192)	n.k.	male	nil	minor	n.k.
M9 (220692)	33	female	30+ cigs/ day	n.k.	none
M10 (220991)	66	male	nil	minor	none
M11 (060690)	62	female	10 cigs/ day	occasional	none
M12 (260788)	43	female	20 cigs/ day	occasional	none
M13 (190290)	22	female	15 cigs/ day	regular	n.k.
M14 (211091)	42	male	heavy	major	Regular Benzodiazepines. Frequent drug overdoses.
M15 (080590)	48	male	heavy	heavy	n.k.
M16 (080390)	31	male	n.k.	n.k.	none

n.k. = not known

Approximately 1g of liver was taken from each of the 16 human liver samples and microsomes prepared by differential centrifugation as described in chapter 7. The microsomal protein concentration of the preparations was determined by the method of Lowry adapted by Peterson (1977) using bovine serum albumin as standard, as described in chapter 7. A table of the microsomal protein recovery of the livers is given in appendix II.

12.2.2 Biochemical characterisation

Liver microsomes were characterised for cytochromes P450 3A4/5 (n = 16) and 1A1/2, 2B6 and 2E1 (n = 7) activity using the *in vitro* marker substrates nifedipine (100 μ M), ethoxyresorufin (2 μ M), pentoxyresorufin (2 μ M) and *p*-nitrophenol (200 μ M). A-esterase and B-esterase activities of the microsomes (n = 7) were determined using the substrates paraoxon (1mM) and phenylvalerate (1.4mM). Glutathione-S-transferase activity was determined in 7 liver cytosol samples by measuring formation of the DNCB (1mM)/ glutathione (1mM) adduct.

All enzyme activities were measured under pre-determined conditions of linearity with respect to protein concentration and time and used saturating substrate concentrations. Methods for each of the enzyme assays are described in chapter 8.

Nifedipine oxidase activity

Approximately 0.3mg microsomal protein in 1ml KCl/ phosphate buffer (pH 7.4) was incubated with nifedipine for 20min. The reaction was terminated by addition of 400 μ l Tris buffer (pH 9.0) and samples analysed for formation of the metabolite M1 by GC with electron capture detection.

Ethoxyresorufin- and pentoxyresorufin-O-dealkylase activity

Approximately 0.1mg or 0.3mg microsomal protein was incubated with ethoxyresorufin or pentoxyresorufin, respectively, in 1ml phosphate buffer (pH 7.4). Formation of the common metabolite, resorufin, was monitored continuously at 37°C for about 5min and the rate of reaction determined.

p-Nitrophenol hydroxylase activity

Approximately 0.25 - 0.5mg protein in 0.5ml potassium phosphate buffer (pH 6.8) was incubated with *p*-nitrophenol for 30min at 37°C. Formation of 4-nitrocatechol was determined by u.v. detection (250nm) following separation on reverse-phase HPLC.

A-esterase (Paraoxonase) activity

Approximately 0.3mg microsomal protein was incubated with paraoxon in a total volume of 3ml glycine buffer (pH 9.0) held at 30°C. Production of the metabolite, *p*-nitrophenol, was monitored continuously at 412nm for approximately 3min and the rate of reaction determined.

B-esterase (phenylvalerate hydrolase) activity

Liver microsomes were diluted appropriately so that approximately 0.3µg microsomal protein was incubated with phenylvalerate in a total volume of 0.4ml Tris/ EDTA buffer, pH 8.0. Incubations were terminated after 10min and formation of phenol determined photometrically at 510nm.

Glutathione-S-transferase activity

Liver cytosols were diluted appropriately so that approximately 0.03mg protein was incubated with DCNB and reduced glutathione in a total volume of 1ml phosphate buffer (pH 6.5). Formation of the DCNB adduct

was monitored continuously at 340nm whilst held at 37°C and the rate of reaction determined.

12.2.3 Parathion metabolism

Interindividual variations in parathion (200µM and 20µM) metabolism to paraoxon and *p*-nitrophenol was measured for the 16 human liver microsomal preparations. Substrate concentrations were chosen from the rat data to be saturating and approximately at the K_m for the enzyme.

Approximately 0.3mg microsomal protein was incubated for 10min in the presence of NADPH and parathion with Tris/ calcium buffer (pH 7.4) in a total volume of 0.5ml. Control incubations were carried out as described except that either microsomal protein or NADPH was omitted. Formation of *p*-nitrophenol and paraoxon was determined by u.v. detection (290nm) following separation on reverse-phase HPLC, as described in chapter 8.

12.2.3.1 The kinetics of parathion metabolism

The kinetic characteristics of parathion metabolism to paraoxon and *p*-nitrophenol was determined for 3 human liver microsomal preparations, M9, M13 and M16. Incubations were carried out as described above using six parathion concentrations (20µM - 1000µM).

Approximately 0.3mg microsomal protein was incubated for 10min in the presence of NADPH and parathion (20, 50, 70, 200, 500, 1000µM, final concentrations) with Tris/ calcium buffer (pH 7.4) to 0.5ml. The reaction was started by addition of parathion in 2µl aliquots of methanol, as before.

Michaelis-Menten, Lineweaver-Burke and Eadie Hofstee plots of paraoxon and *p*-nitrophenol formation were constructed for each of the human liver microsomes. Data points derived from the Lineweaver-Burke and Eadie Hofstee plots were fitted by least squares linear regression and V_{max} and apparent K_m values determined.

12.2.3.2 Inhibition of parathion metabolism

Inhibitors

Microsomal liver preparations M15 and M16 were incubated with parathion (20 μ M and 200 μ M) and the inhibitors naringenin, troleandomycin, quercetin ketoconazole and quinidine. Microsomal liver preparations M9 and M11 were incubated with parathion (20 μ M and 200 μ M) and the inhibitors metyrapone, α -naphthoflavone, diethyldithiocarbamate (DEDC) and tolbutamide. Liver microsomes (M11, M12 and M16) were incubated with parathion (200 μ M) and sulphaphenazole. Inhibitor concentrations were 100 μ M, except sulphaphenazole (10 μ M).

Inhibitors (5 μ l) or vehicle (5 μ l DMSO or methanol, control) were added to incubation tubes containing NADPH (0.3mg) and microsomal protein (approx. 0.4mg) and made up to 0.5ml with Tris/ calcium buffer (pH 7.4). The tubes were pre-incubated with inhibitors or vehicle for 10min prior to starting the reaction by the addition of parathion (20 μ M and 200 μ M, final concentration). The reaction was terminated after 10min by addition of 0.5ml perchloric acid (20%, w/v) and formation of *p*-nitrophenol and paraoxon determined by HPLC with u.v. detection, as described previously.

The nature of parathion inhibition by naringenin

Competitive or non-competitive inhibition can be evaluated through the use of the Dixon plot, which calculates the enzyme inhibitor constant, K_i . A plot of $1/v$ against inhibitor concentration, at constant substrate concentration, gives a straight line. When this is done at two or more different substrate concentrations, an intersection of the lines to the left of the vertical axis indicates a competitive inhibitor. The intersection point is equivalent to $-K_i$, the inhibitor constant. If the lines are parallel, the inhibition is non-competitive.

The nature of naringenin inhibition of the paraoxon formed from parathion was assessed by use of the Dixon plot. In this study human liver microsomes (M11) were pre-incubated with a range of naringenin concentrations (5-100 μ M) at three concentrations of parathion (20, 100 and 200 μ M). The data points were fitted by least squares linear regression and the K_i determined.

12.2.4 Inhibition of paraoxonase activity

These experiments were designed to investigate the involvement of A-esterases in *p*-nitrophenol formation from parathion and paraoxon.

Liver microsomes (M9, M15 and M16) were preincubated for 10min in the presence of EDTA (ethylenediaminetetra-acetic acid, 5mM) and 50mM Tris buffer pH 7.4 (without calcium) to 0.5ml, before addition of parathion (20 μ M or 200 μ M) or paraoxon (1 μ M, 3 μ M or 1000 μ M). Control incubations were carried out in parallel in 50mM Tris/ 1mM CaCl_2 buffer, pH 7.4 (no EDTA). Paraoxon and *p*-nitrophenol levels were measured by reverse-phase HPLC with u.v. detection, as described in chapter 8.

12.2.5 Correlations between parathion metabolism and P450 marker activities

Correlation coefficients were calculated for the previously defined (chapter 12.1.2) rates of nifedipine oxidation, ethoxyresorufin-*O*-dealkylation, pentoxyresorufin-*O*-dealkylation, *p*-nitrophenol hydroxylation, paraoxon hydrolysis and phenylvalerate hydrolysis with the rates of formation of paraoxon and *p*-nitrophenol from parathion (20 μ M and 200 μ M) (chapter 12.1.3).

The correlation coefficient (*r*) was calculated by least squares linear regression fit and from this values of *t* were determined. The data were also analysed by non-parametric statistics, the Spearman rank correlation coefficient. Values at $p < 0.05$ or less were considered to be significant.

12.2.6 Studies with P450-expressed microsomes

Commercially (Gentest) available microsomes from cell lines expressing human P450s 3A4, 3A5, 1A1, 2B6, 2E1, 2C8 and 2C9-Arg₁₄₄ were assessed for their capacity to metabolise parathion and nifedipine (P450s 3A4 and 3A5 only).

Microsomes (20pmol P450/ incubation) were incubated for 2h in 0.5ml 100mM phosphate buffer (pH 7.4) with NADPH (0.6mg/ml) and parathion (200 μ M), as described previously. Paraoxon and *p*-nitrophenol formation measured by HPLC with u.v. detection, as before. Microsomes expressing P450s 3A4 and 3A5 (20pmol P450) were also incubated with nifedipine (100 μ M) and formation of M1 measured by GC with electron capture, as

described previously. Appropriate control microsomes (vector only) were incubated under the same conditions.

12.3 Results

12.3.1 Biochemical characterisation

Interindividual variations for the enzymes measured in human liver microsomes are given in table 12.2. Individual data are given in appendix III.

Table 12.2 Enzyme activities for the panel of human liver microsomal preparations.

Enzyme reaction	n	Enzyme activity	
		Range (median)	Mean \pm sem
Nifedipine oxidation	16	0.055 - 5.72 (1.72) nmol/ min/ mg protein	2.57 \pm 0.41
Ethoxyresorufin-O-dealkylation	7	10.0 - 124.4 (41.7) pmol/ min/ mg protein	54.7 \pm 14.7
Pentoxyresorufin-O-dealkylation	7	n.d. - 11.0 (4.7) pmol/ min/ mg protein	5.2 \pm 2.0
<i>p</i> -Nitrophenol hydroxylation	7	0.662 - 1.32 (0.797) nmol/ min/ mg protein	0.913 \pm 0.095
Paraoxon hydrolysis	7	4.6 - 16.0 (7.3) nmol/ min/ mg protein	8.8 \pm 1.6
Phenylvalerate hydrolysis	7	6.34 - 14.9 (10.3) μ mol/ min/ mg protein	10.8 \pm 1.15
DNCB/ glutathione conjugation	7	553 - 1190 (892) nmol/ min/ mg protein	880 \pm 93.6

n.d. = below the limit of detection (0.1pmol/ min)

DNCB is 2, 4-dinitro-1-chlorobenzene

The rate of nifedipine oxidation, to the metabolite M1, ranged from 0.055 to 5.72 (median, 1.72) nmol/ min/ mg protein for the sixteen human liver microsomes which was similar to that found by other workers (Iribarne *et al*, 1996), although livers M3 and M4 were at the extreme of this range. The frequency distribution histogram for the formation of M1 from nifedipine (figure 12.1) clearly shows these two outliers.

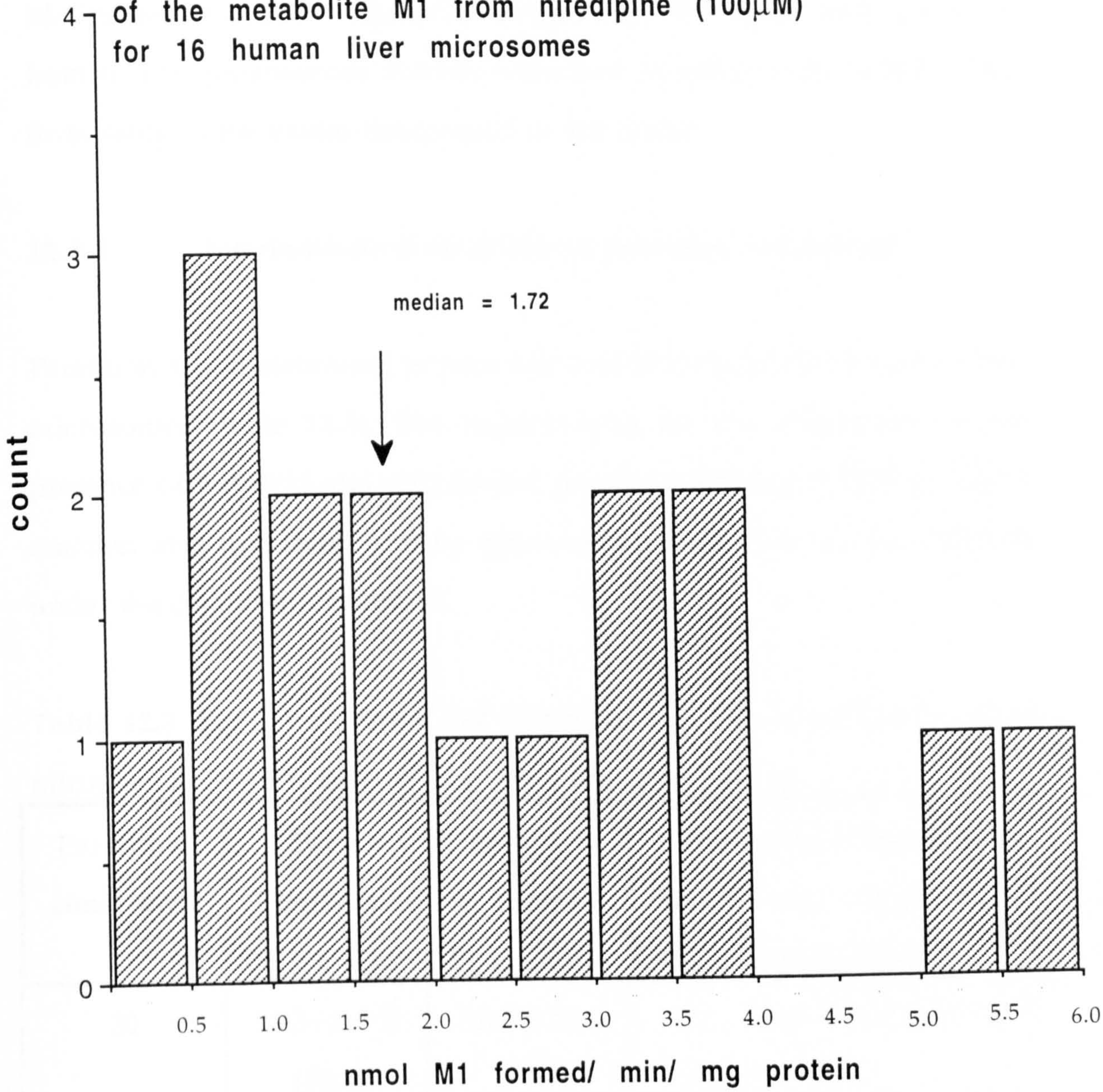
Ethoxyresorufin- and pentoxyresorufin-O-dealkylase activities for the panel of human liver microsomes were 10.0 - 124.4 and n.d. - 11.0 pmol/ min/ mg protein, respectively. Activities quoted in the literature for ethoxyresorufin-O-dealkylase activity, 160 ± 25 pmol/ min/ mg protein, (Weaver *et al*, 1994) compare favourably. Literature quotes for pentoxyresorufin-O-dealkylase activity range from 2.8 ± 2.1 (Waziers *et al*, 1989) to 5.0 ± 5.0 pmol/ min/ mg protein (Shimada *et al*, 1997). Pentoxyresorufin-O-dealkylase activity was about ten fold lower than that obtained for the O-dealkylation of ethoxyresorufin.

The range for formation of 4-nitrocatechol from *p*-nitrophenol was 0.662 - 1.32 nmol/ min/ mg protein which was similar to the rate reported by Zerilli *et al* (1997), 2.38 ± 1.55 nmol/ min/ mg protein.

Concerning glutathione-S-transferase activity, non-enzymatic formation of the DNCB adduct was less than 5% of that produced enzymatically, although this value tended to increase during the course of the day as reagents deteriorated. The range determined in this study, 553 - 1190 nmol/ min/ mg cytosolic protein, for a small population of human livers was similar to the range of 480 - 2820 nmol/ min/ mg protein reported by Temellini *et al*, 1995 for 93 livers of mixed gender.

Figure 12.1

Frequency distribution histogram for the formation of the metabolite M1 from nifedipine (100 μ M) for 16 human liver microsomes



Human liver microsomes were characterised for paraoxonase activity at pH 9.0 since preliminary experiments (see chapter 8) had shown that this provided optimal conditions. There is a paucity of literature information concerning liver esterase activities, particularly for human data. However, McCracken *et al* (1993) reported a range of 25 - 80 nmol/ min/ g liver for human liver paraoxonase activity measured at optimal pH, which relates favourably to the values determined in this study.

12..3.2 Interindividual variations in parathion metabolism

Parathion was metabolised to paraoxon and *p*-nitrophenol by human liver microsomes (table 12.3). The reaction was entirely dependent on the presence of NADPH and microsomal protein indicating a P450 mediated reaction and that there was no spontaneous hydrolysis of the substrate under the conditions described.

Table 12.3 Parathion (20µM and 200µM) metabolism to paraoxon and *p*-nitrophenol by 16 human liver microsomal preparations.

Parathion conc. (µM)	Paraoxon formation (pmol/ min/ mg protein)		<i>p</i> -Nitrophenol formation (pmol/ min/ mg protein)	
	Range (median)	Mean ± sem	Range (median)	Mean ± sem
20	23.3 - 199.3 (70.8)	90.3 ± 12.5	321.1 - 769.2 (513.1)	538.0 ± 26.8\$
200	18.7 - 310.3 (117.3)	153.2* ± 21.3	406.2 ± 778.3 (558.1)	583.2 ± 26.3@

*p<0.05 compared to paraoxon formation from 20µM parathion

\$p<0.05 compared to paraoxon formation from 20µM parathion

@p<0.05 compared to paraoxon formation from 200µM parathion

(statistics by paired t-test)

Paraoxon was formed from 20 μ M and 200 μ M parathion at 23.3-199.3 (70.8, median) and 18.7-310.3 (117.3, median) pmol/ min/ mg protein, respectively, which indicates a 9-fold and 16-fold interindividual variation in activation capacity by microsomes from 16 human livers (table 12.3). *p*-Nitrophenol was also formed at 321.1-769.2 (513.1, median) and 406.2-778.3 (558.1, median) pmol/ min/ mg protein, indicating a twofold interindividual variation regardless of substrate concentration.

Paraoxon levels were higher at 200 μ M parathion compared to the lower concentration while *p*-nitrophenol levels were similar at both substrate concentrations. *p*-Nitrophenol formation was greater than paraoxon at both parathion concentrations. The ratio of paraoxon to *p*-nitrophenol from parathion (20 μ M and 200 μ M) was 0.036-0.468 (median 0.154) and 0.036-0.593 (median 0.257), respectively. Individual paraoxon/ *p*-nitrophenol ratios for the sixteen liver microsomes are given in figure 12.2 (20 μ M parathion) and figure 12.3 (200 μ M parathion).

12.3.3 The kinetic parameters of parathion metabolism

The K_m and V_{max} values for paraoxon and *p*-nitrophenol formation derived from Lineweaver-Burke plots are given in table 12.4 for 3 human liver microsomal preparations.

Figure 12.2
Paraoxon and p-nitrophenol formation from parathion (20μM)
for 16 human liver microsomes.
Individual paraoxon/ p-nitrophenol ratios (vertical orientation)
are given in ascending order

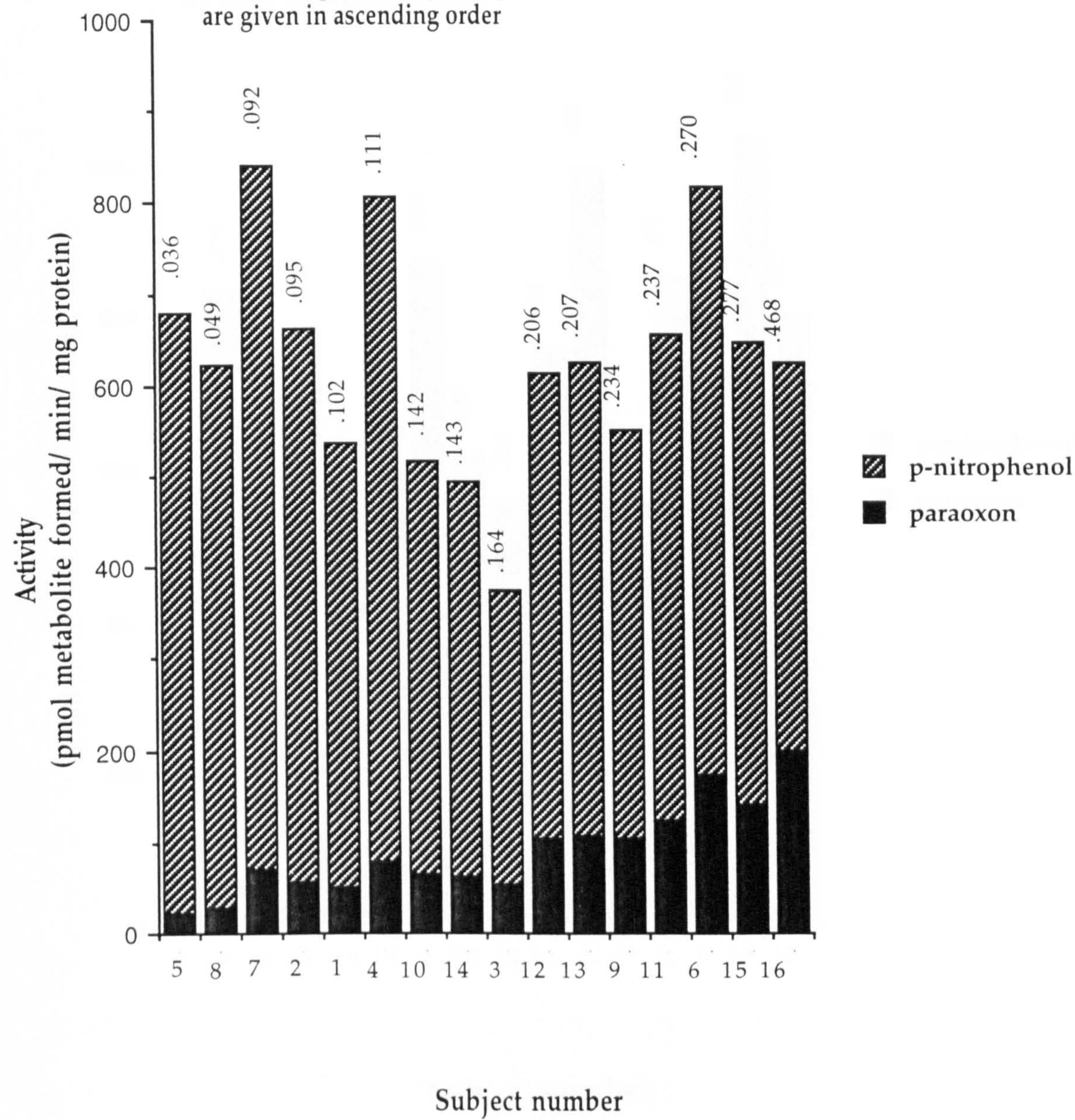


Figure 12.3
 Paraoxon and p-nitrophenol formation from parathion (200 μ M)
 for 16 human liver microsomes.
 Individual paraoxon/ p-nitrophenol ratios (vertical orientation)
 are given in ascending order

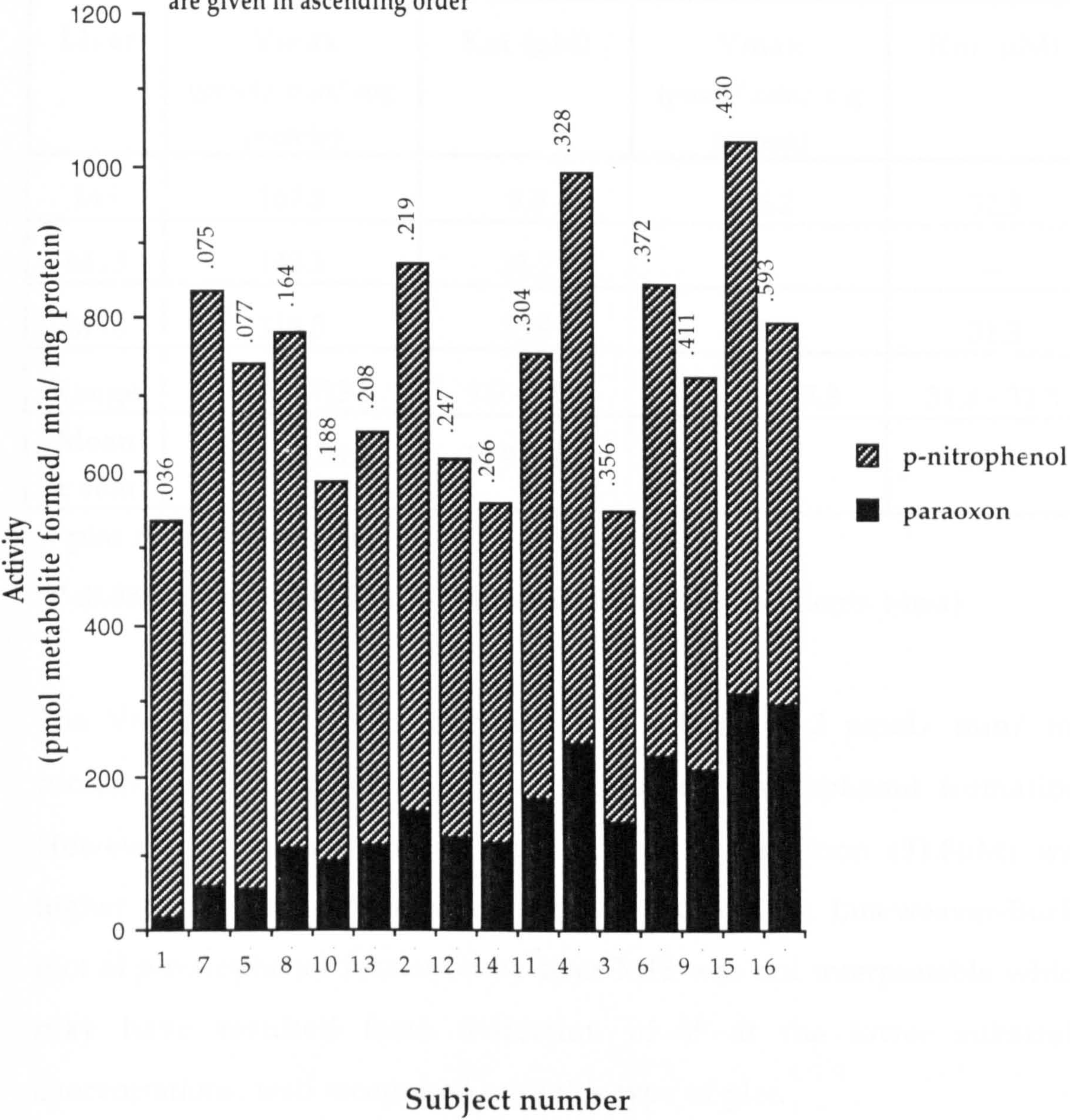


Table 12.4 Kinetic parameters for paraoxon and *p*-nitrophenol formation from parathion by 3 human liver microsomal preparations. Values were derived from Lineweaver-Burke plots of the data.

	Paraoxon formation		<i>p</i> -Nitrophenol formation	
Liver	Vmax (pmol/ min/ mg protein)	Km (μM)	Vmax (pmol/ min/ mg protein)	Km (μM)
M9	167.8	9.0	206.2	32.3
M13	143.1	13.7	--	--
M16	313.5	15.9	255.8	31.3
Range	167.8 - 313.5	9.0 - 15.9	206.2 - 255.8	31.3 - 32.3
Mean ± sem	208.1 ± 53.2	12.9 ± 2.0	231.0	31.8*

-- plot not interpretable

*P<0.05, compared to Km for paraoxon formation (students t-test).

The Vmax for paraoxon formation was 208.1 ± 53.2 pmol/ min/ mg protein and 231.0 pmol/ min/ mg protein for *p*-nitrophenol formation. However, the apparent Km for *p*-nitrophenol formation (31.8μM) was higher than that for paraoxon, 12.9μM ± 2.0μM. The Lineweaver-Burke plot of *p*-nitrophenol formation by liver M13 was not interpretable which may have resulted from distortion of *V* at the lower substrate concentrations, well recognised with this type of plot.

Michaelis-Menten, Lineweaver-Burke and Eadie Hofstee plots of paraoxon and *p*-nitrophenol formation by liver M9 are given in figures 12.4, 12.5, 12.6 and 12.7, respectively. Visual inspection of the Eadie Hofstee plot of paraoxon and *p*-nitrophenol formation suggested monophasic kinetics over the substrate range studied. The Eadie-Hofstee plot for liver M9 produced Vmax values of 167.7 and 203.3 pmol/ min/ mg protein for

Figure 12.4

A Michaelis-Menten plot of paraoxon and p-nitrophenol formation from varying concentrations of parathion by human liver microsomes M9

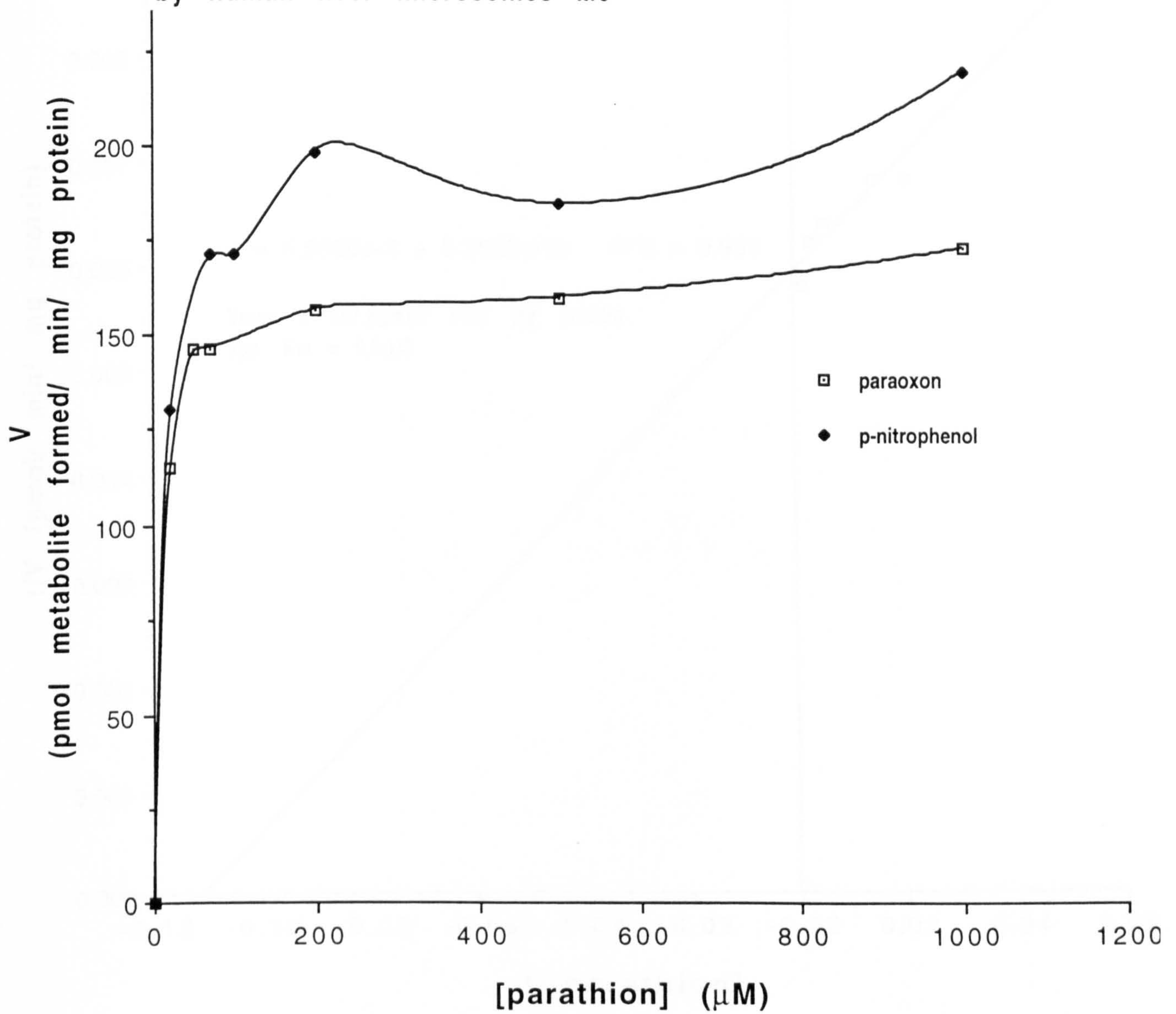
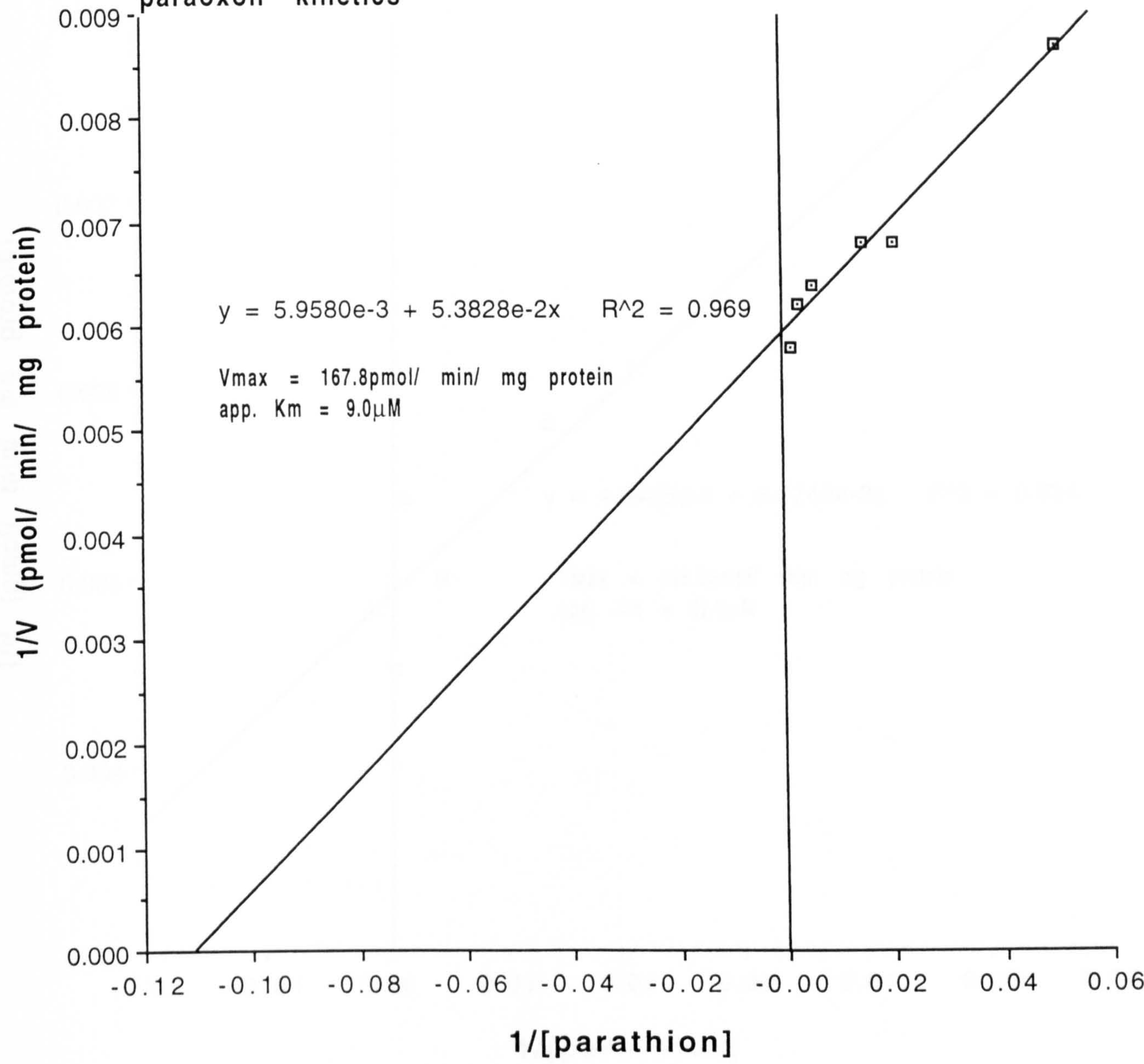
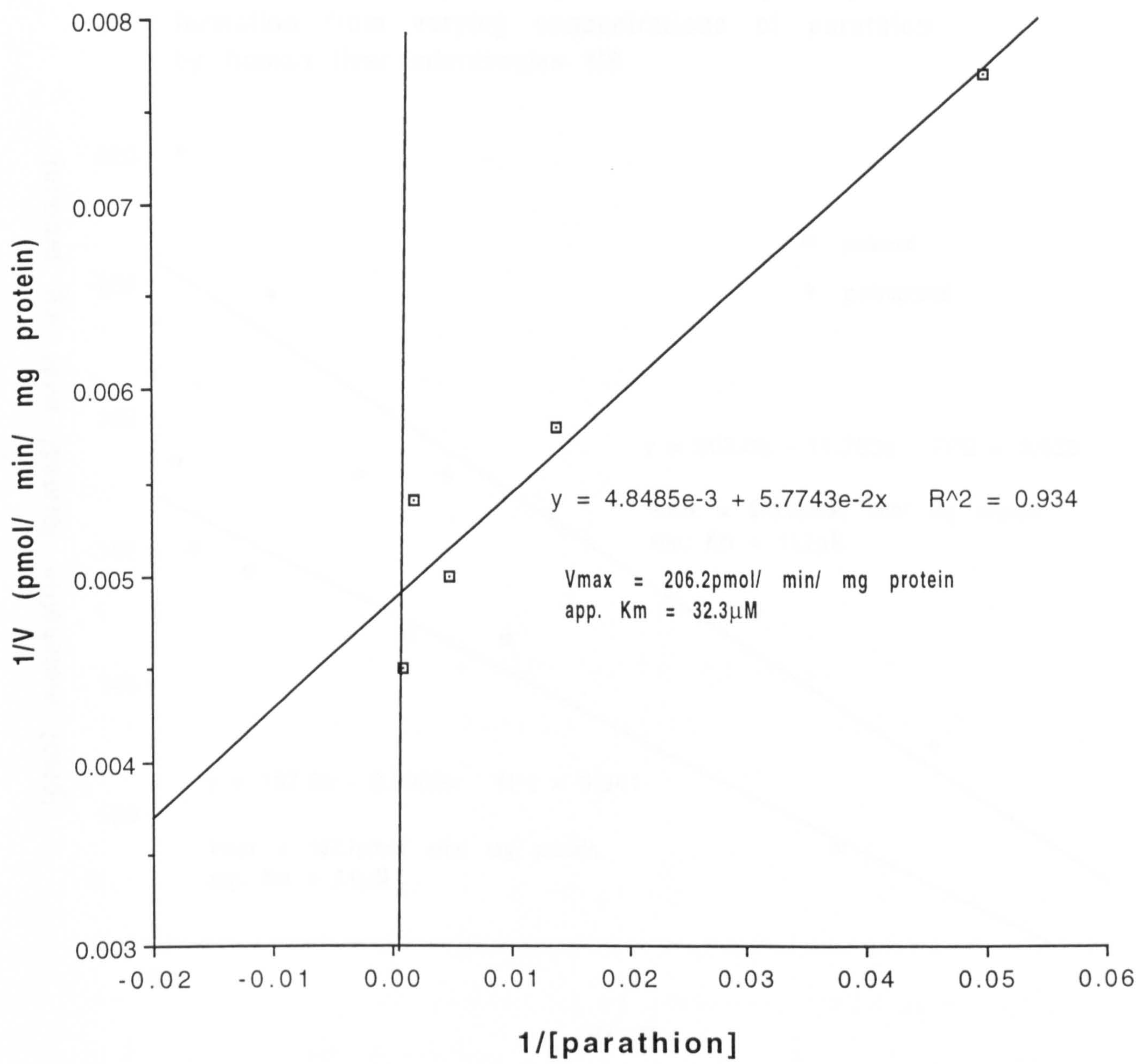


Figure 12.5
Lineweaver-Burke plot for human liver microsomes M9:
paraoxon kinetics



Data fitted by least squares linear regression

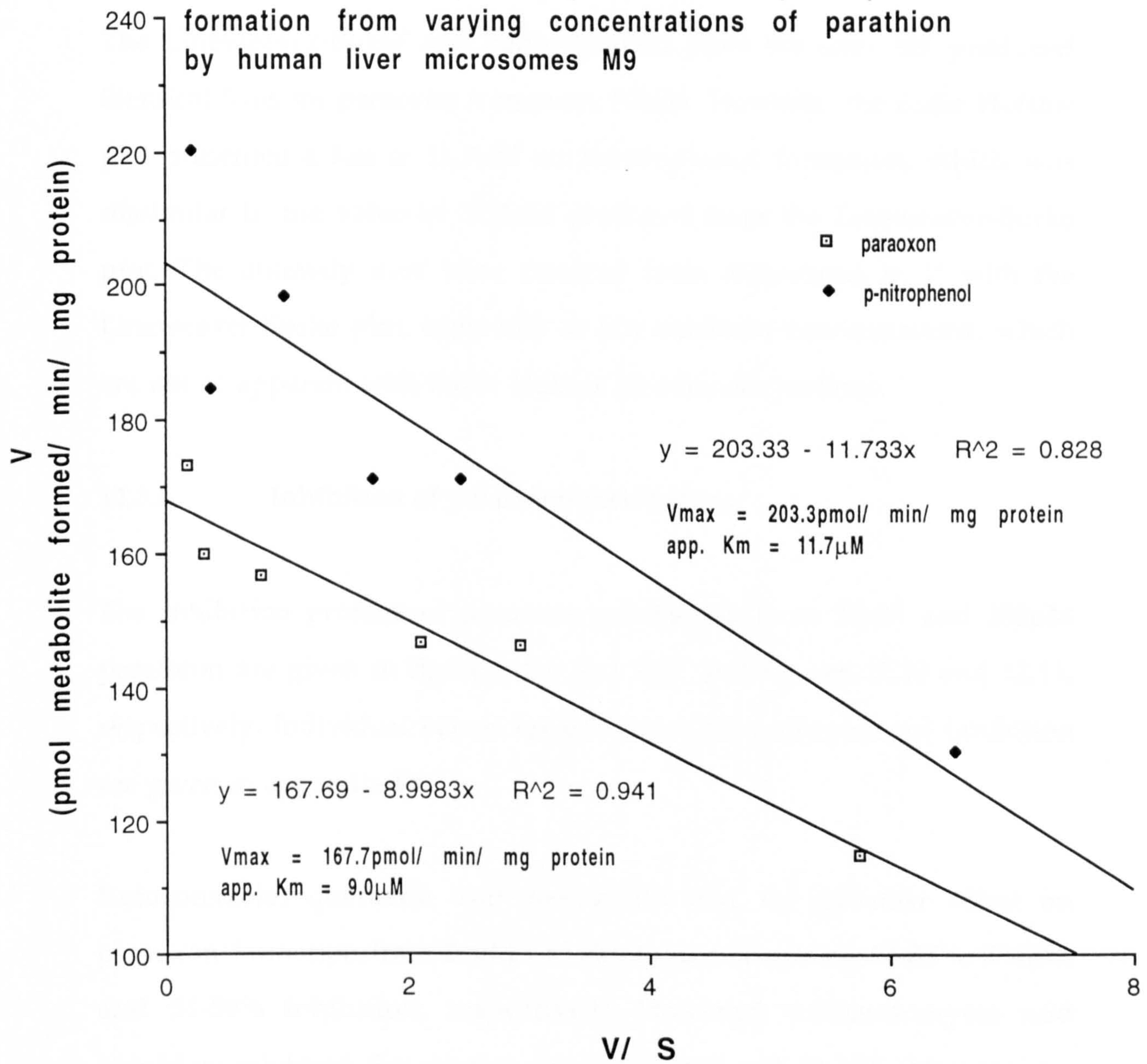
Figure 12.6
Lineweaver-Burke plot for human liver microsomes M9:
p-nitrophenol kinetics



Data fitted by least squares linear regression

Figure 12.7

An Eadie Hofstee plot of paraoxon and p-nitrophenol formation from varying concentrations of parathion by human liver microsomes M9



Data fitted by least squares linear regression

paraoxon and *p*-nitrophenol formation, respectively, which is in complete agreement with those derived from the Lineweaver-Burke plot (table 12.4).

The Lineweaver-Burke and Eadie Hofstee plots for liver M9 produced identical K_m s for paraoxon formation, $9.0\mu\text{M}$. However, the Eadie Hofstee plot presented a K_m of $11.7\mu\text{M}$ for *p*-nitrophenol formation, which was dissimilar to the value of $32.3\mu\text{M}$ produced from the Lineweaver-Burke plot. The anomaly may have resulted from distortions in V with the Lineweaver-Burke plot, especially at low substrate concentrations, which are not so apparent with Eadie Hofstee plot transformations.

12.3.4 Inhibition of parathion metabolism

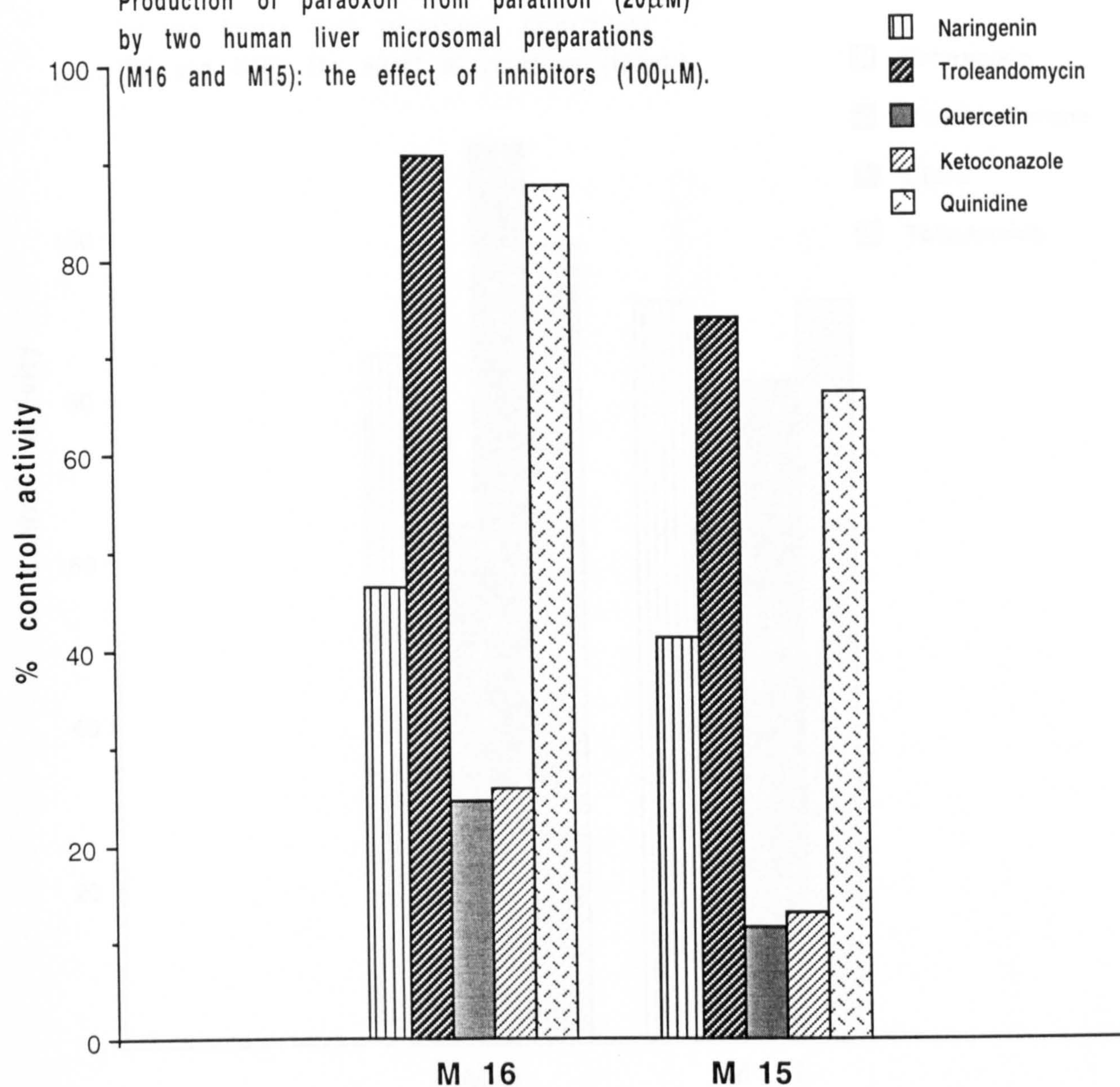
The inhibition profiles of paraoxon production from $20\mu\text{M}$ and $200\mu\text{M}$ parathion are given in figures 12.8 and 12.9, and figures 12.10 and 12.11, respectively. Individual values for paraoxon and *p*-nitrophenol inhibition are given in appendix IV.

Ketoconazole, quercetin and naringenin had the greatest effect on paraoxon formation from $20\mu\text{M}$ parathion, demonstrating 74-87%, 75-88% and 54-59% inhibition, respectively. However, troleandomycin and quinidine inhibited the reaction by only 9-26% and 12-33%. Metyrapone, diethyldithiocarbamate and tolbutamide did not greatly inhibit parathion ($20\mu\text{M}$) activation but α -naphthoflavone produced 35-36% inhibition.

Troleandomycin had slightly less inhibitory effect (10-15%) for paraoxon formation at the high ($200\mu\text{M}$) compared to the low ($20\mu\text{M}$) parathion concentration. Ketoconazole, quercetin and naringenin inhibited

Figure 12.8

Production of paraoxon from parathion ($20\mu\text{M}$)
by two human liver microsomal preparations
(M16 and M15): the effect of inhibitors ($100\mu\text{M}$).

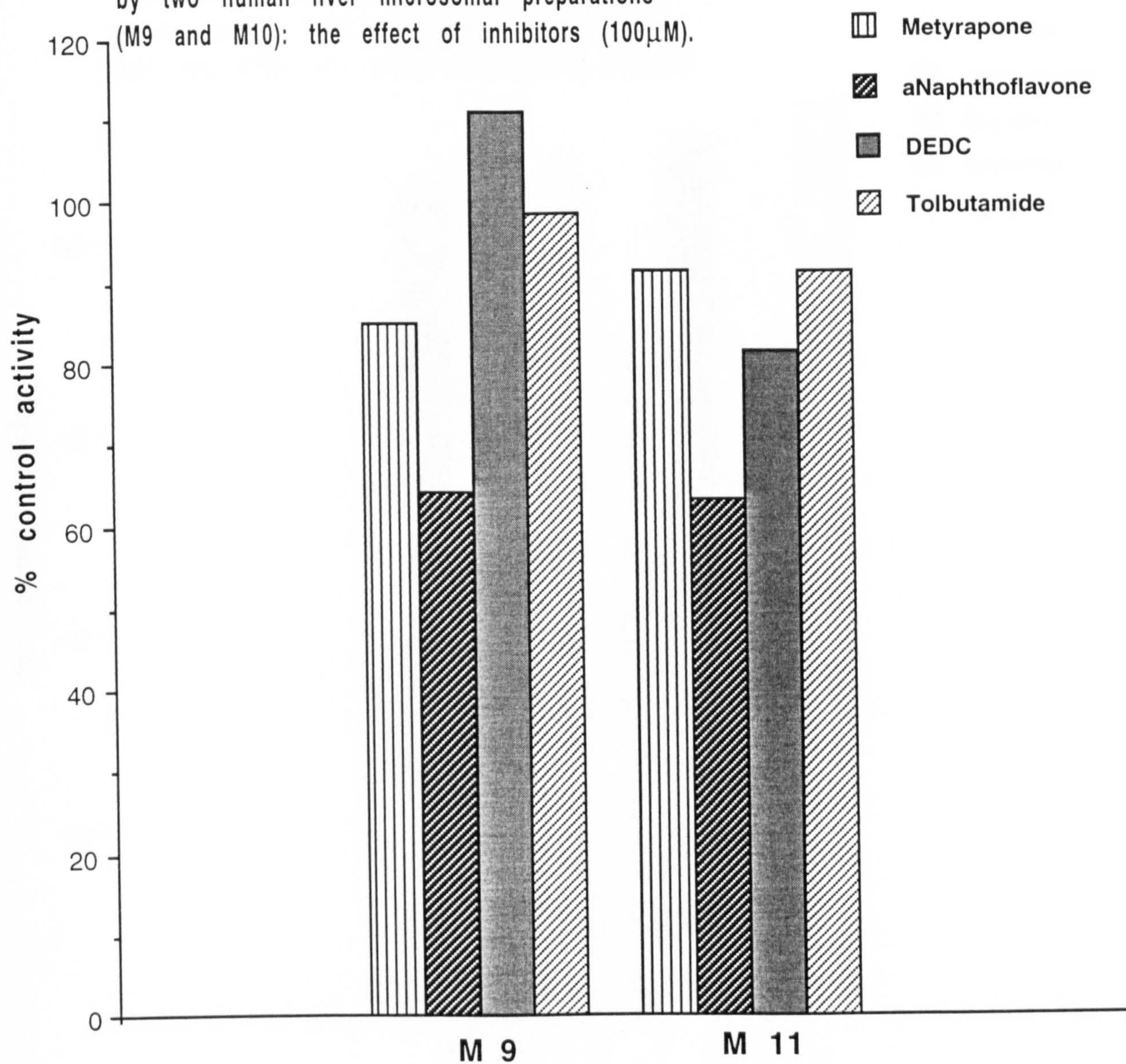


Values are the mean of duplicate determinations

Control (100%) values are given in appendix III

Figure 12.9

Production of paraoxon from parathion ($20\mu\text{M}$)
by two human liver microsomal preparations
(M9 and M10): the effect of inhibitors ($100\mu\text{M}$).

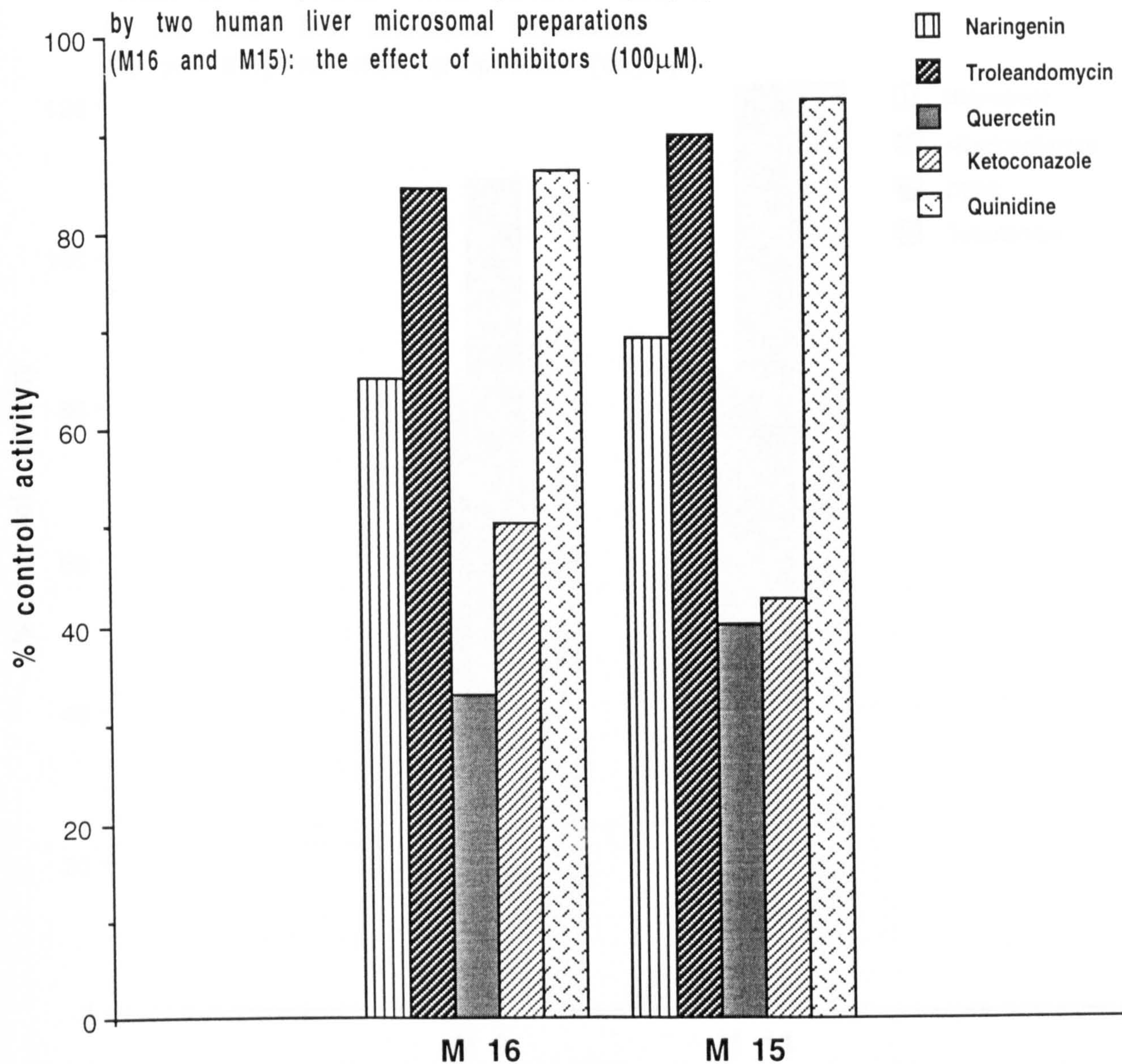


Values are the mean of duplicate determinations

Control (100%) values are given in appendix III

Figure 12.10

Production of paraoxon from parathion (200 μ M)
by two human liver microsomal preparations
(M16 and M15): the effect of inhibitors (100 μ M).



Values are the mean of duplicate determinations

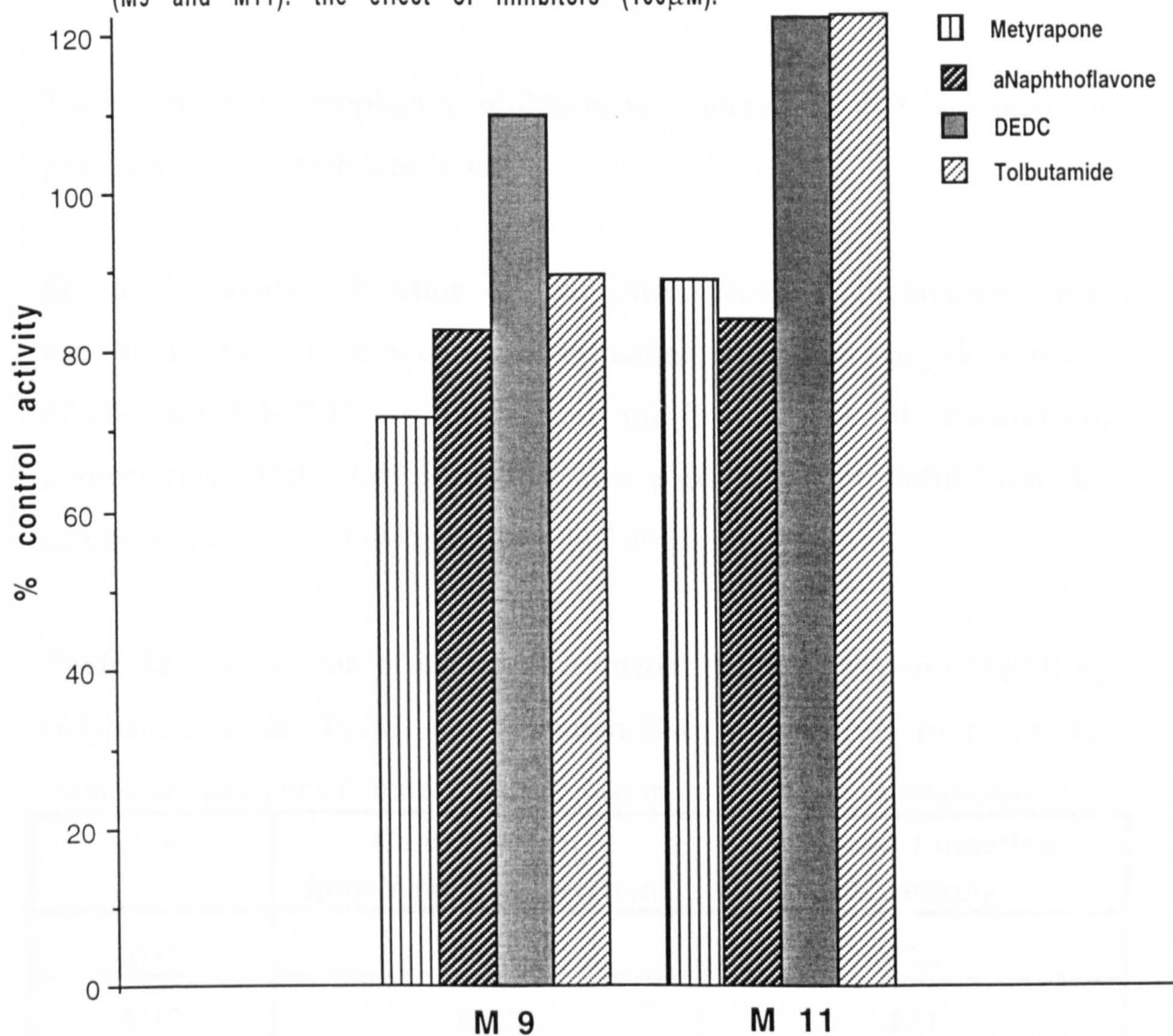
Control (100%) values are given in appendix III

Figure 12.11

Production of paraoxon from parathion ($200\mu\text{M}$)

by two human liver microsomal preparations

(M9 and M11): the effect of inhibitors ($100\mu\text{M}$).



Values are the mean of duplicate determinations

Control (100%) values are given in appendix III

parathion (200µM) activation to the extent of 50-57%, 60-67% and 31-35%, respectively. Metyrapone (11-28%), α-naphthoflavone (16-17%), diethyldithiocarbamate (10-22% increase in activity) tolbutamide (10% decrease - 23% increase in activity) and quinidine (6-13%) produced little inhibition of parathion activation at 200µM.

The profile of *p*-nitrophenol inhibition was similar to that observed for paraoxon for all inhibitors tested.

Sulphaphenazole inhibition of parathion (200µM) metabolism was variable for the 3 liver microsomes examined. Inhibition ranged from 2.8-57.9% and 1.4-77.1% for paraoxon and *p*-nitrophenol formation, respectively. Data for paraoxon and *p*-nitrophenol inhibition by sulphaphenazole are given in tables 12.5 and 12.6.

Table 12.5 Inhibition of paraoxon formation from parathion (200µM) by sulphaphenazole (10µM) for 3 human liver microsomal preparations. Values are percent of control activity (mean of duplicate estimations).

Liver	Control activity (pmol/ min/ mg protein)	Paraoxon formation (% control)
M11	210.2	97.2
M12	150.3	42.1
M16	315.7	88.0
Range	150.3 - 315.7	42.1 - 97.2
Mean ± sem	225.4 ± 48.3	75.8 ± 13.9

Table 12.6 Inhibition of *p*-nitrophenol formation from parathion (200μM) by sulphaphenazole (10μM) for 3 human liver microsomal preparations. Values are percent of control activity (mean of duplicate estimations).

Liver	Control activity (pmol/ min/ mg protein)	<i>p</i> -Nitrophenol formation (% control)
M11	543.1	98.6
M12	560.2	22.9
M16	462.9	98.0
Range	462.9 - 560.2	22.9 - 98.6
Mean ± sem	522.1 ± 30.0	73.2 ± 25.1

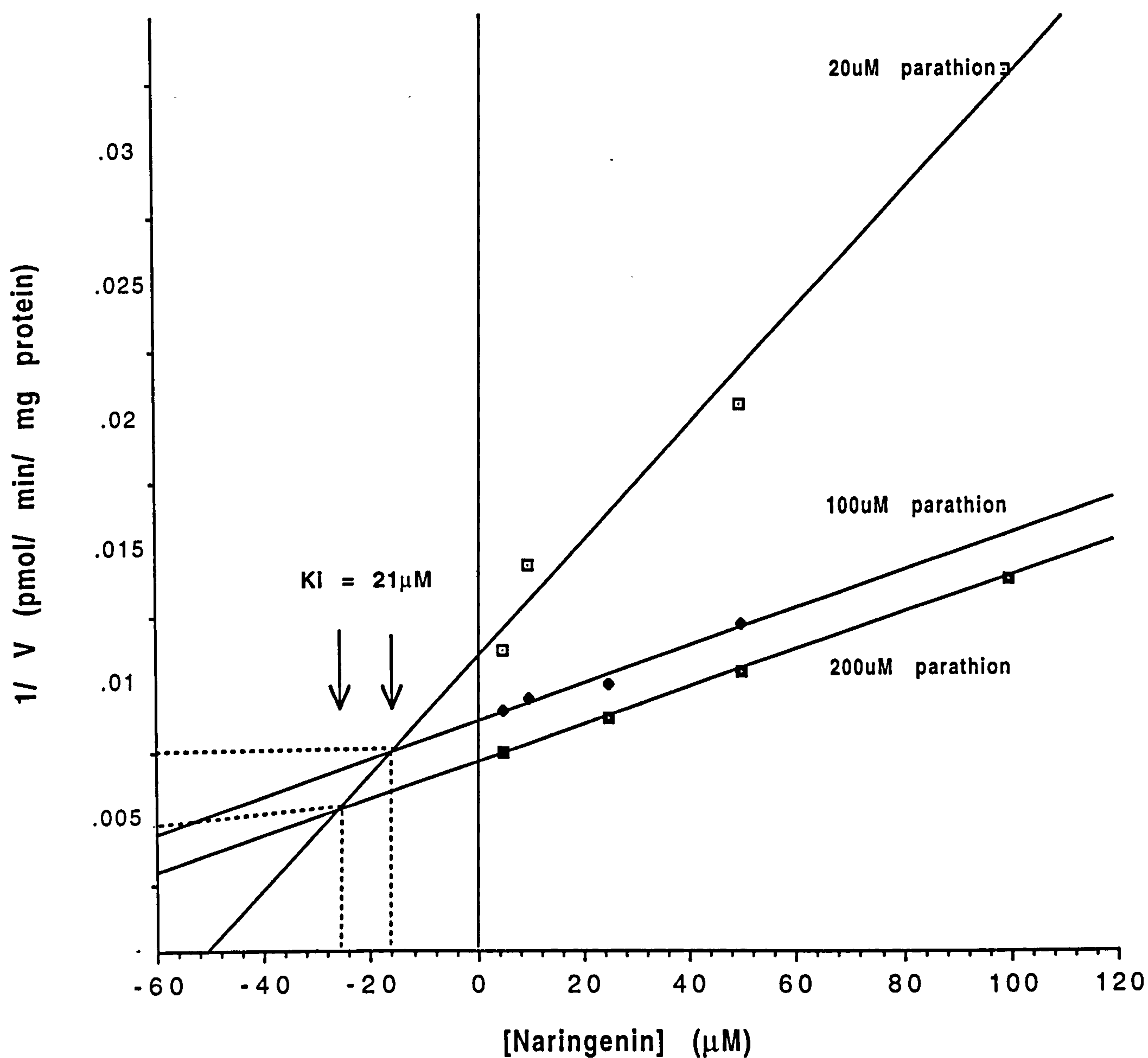
A *K_i* value of 21μM for naringenin inhibition of parathion was estimated from the Dixon plot (figure 12.12). The plot suggested competitive inhibition at low (20μM) substrate concentration and non-competitive inhibition at the higher (100μM and 200μM) concentrations where the plot produced lines which were parallel.

12.3.5 Inhibition of paraoxonase activity

Incubations of liver microsomes with parathion (20μM or 200μM) in the presence of 5mM EDTA did not affect the levels of paraoxon and *p*-nitrophenol formed compared to control. *p*-Nitrophenol was not detected following incubation with the concentrations of paraoxon (1μM or 3μM) produced from 20μM or 200μM parathion whether EDTA was present or not. However, incubations with saturating concentration of paraoxon (1mM) and EDTA produced 72% of control *p*-nitrophenol levels. Results are given in table 12.7.

Figure 12.12

Dixon plot illustrating the inhibitory effect of naringenin on parathion activation to paraoxon by human liver microsomes (M11)



Data fitted by least squares linear regression

Table 12.7 The effect of EDTA (5mM) on the metabolism of parathion and paraoxon by human liver microsomes. Values are the mean \pm s.e.m. (n=3).

Substrate	Enzyme activity	
	Control	EDTA
Parathion (20μM)	148.2 \pm 27.6 ^a	149.4 \pm 23.8 ^a
	461.0 \pm 25.1 ^b	490.2 \pm 29.3 ^b
Parathion (200μM)	272.1 \pm 31.2 ^a	289.4 \pm 45.4 ^a
	577.0 \pm 72.1 ^b	579.3 \pm 93.4 ^b
Paraoxon (1μM)	n.d. ^b	n.d. ^b
Paraoxon (3μM)	n.d. ^b	n.b. ^b
Paraoxon (1000μM)	1765 \pm 333 ^b	1270 \pm 240 ^{b*}

^a pmol paraoxon formed min⁻¹ mg protein⁻¹

^b pmol *p*-nitrophenol formed min⁻¹ mg protein⁻¹

n.d. below the limit of detection (10pmol *p*-nitrophenol)

*p<0.05, compared to control activity

12.3.6 Correlations between parathion metabolism and P450 marker activities

Correlations between the enzyme activities measured and paraoxon and *p*-nitrophenol formation from parathion (200μM) are given in table 12.8. There was significant (p<0.02) correlation between activation of parathion (200μM) to paraoxon and oxidation of nifedipine (100μM) to M1 (figure 12.13). However, correlation between parathion metabolism at low substrate (20μM) and nifedipine oxidation (figure 12.14) did not reach statistical significance, but did achieve significance (p<0.05) when the two outliers were omitted (r = 0.571 and Spearman rank correlation coefficient = 0.495). Correlation between *p*-nitrophenol formation from parathion (200μM) and nifedipine oxidation was also significant (p<0.05) when the two outliers were omitted (r = 0.560 and Spearman rank correlation coefficient = 0.604).

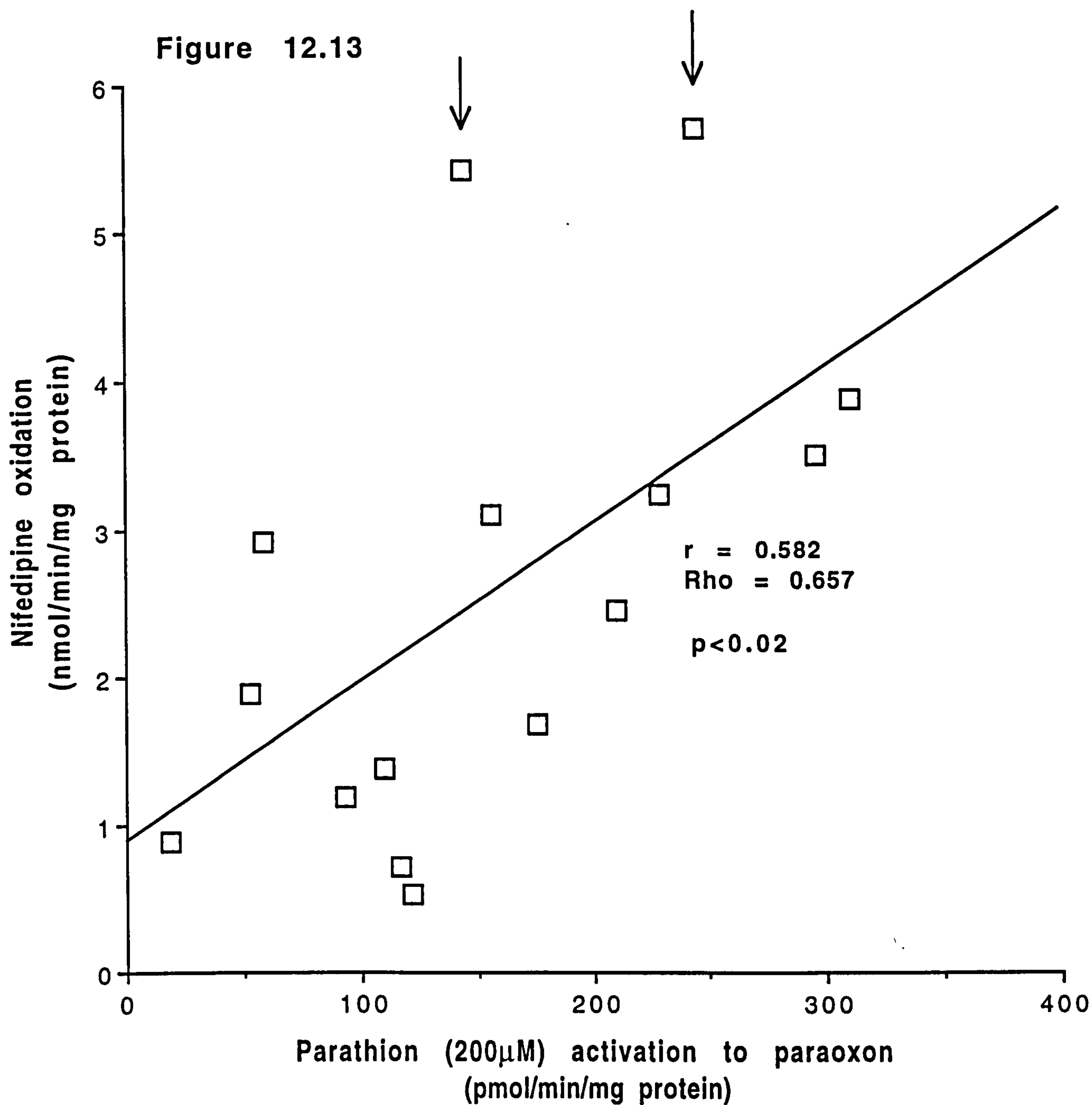


Figure 12.13

Correlation between the activation of parathion (200µM) and nifedipine (100µM) oxidation by 16 human liver microsomal preparations.

Arrows denote the two outliers.

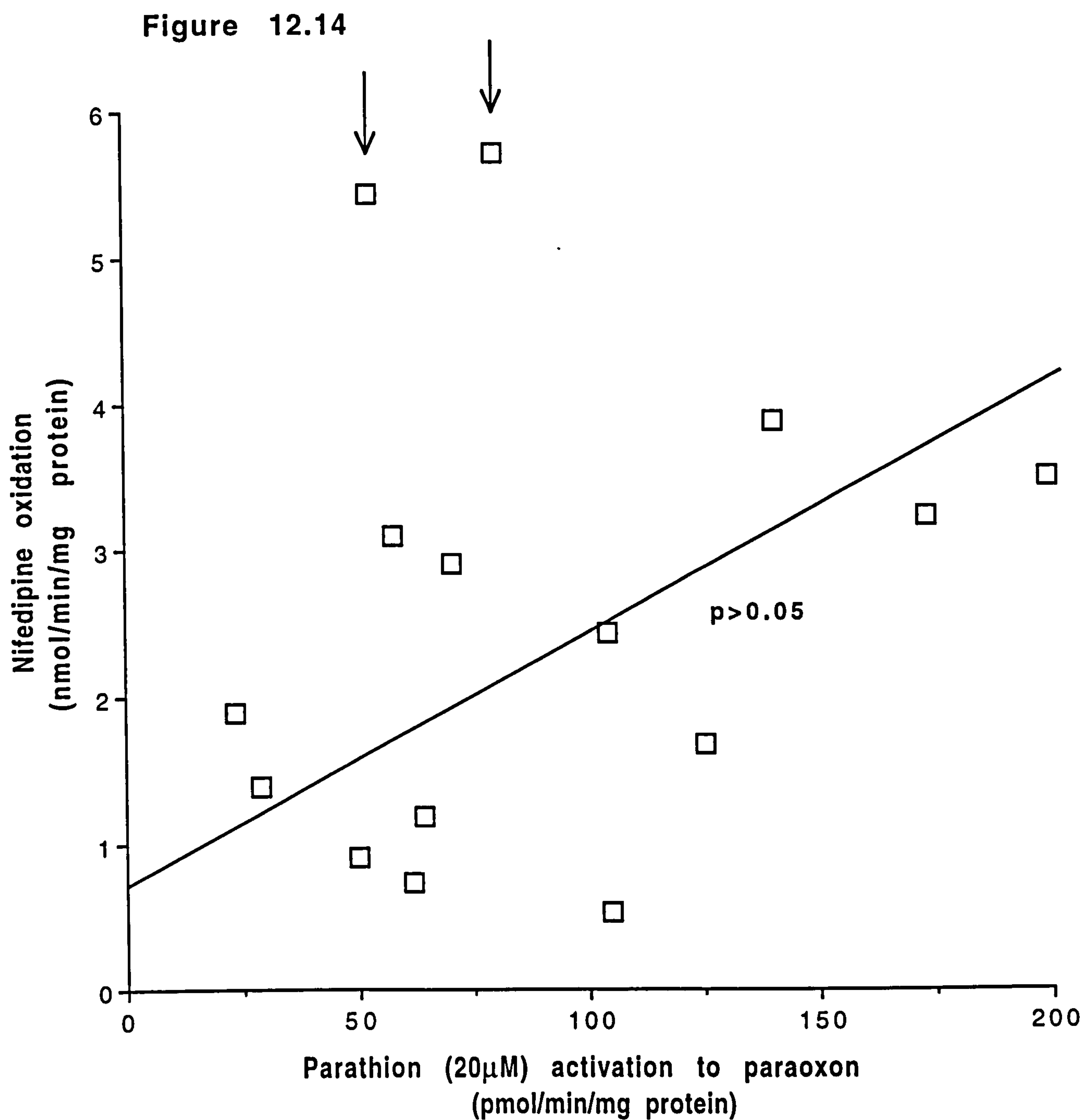


Figure 12.14

Correlation between the activation of parathion (20µM) and nifedipine (100µM) oxidation by 16 human liver microsomal preparations.

Arrows denote the two outliers.

Figure 12.15

Correlation between the activation of parathion (200 μ M) to paraoxon and ethoxyresorufin-O-dealkylation for 7 human liver microsomes

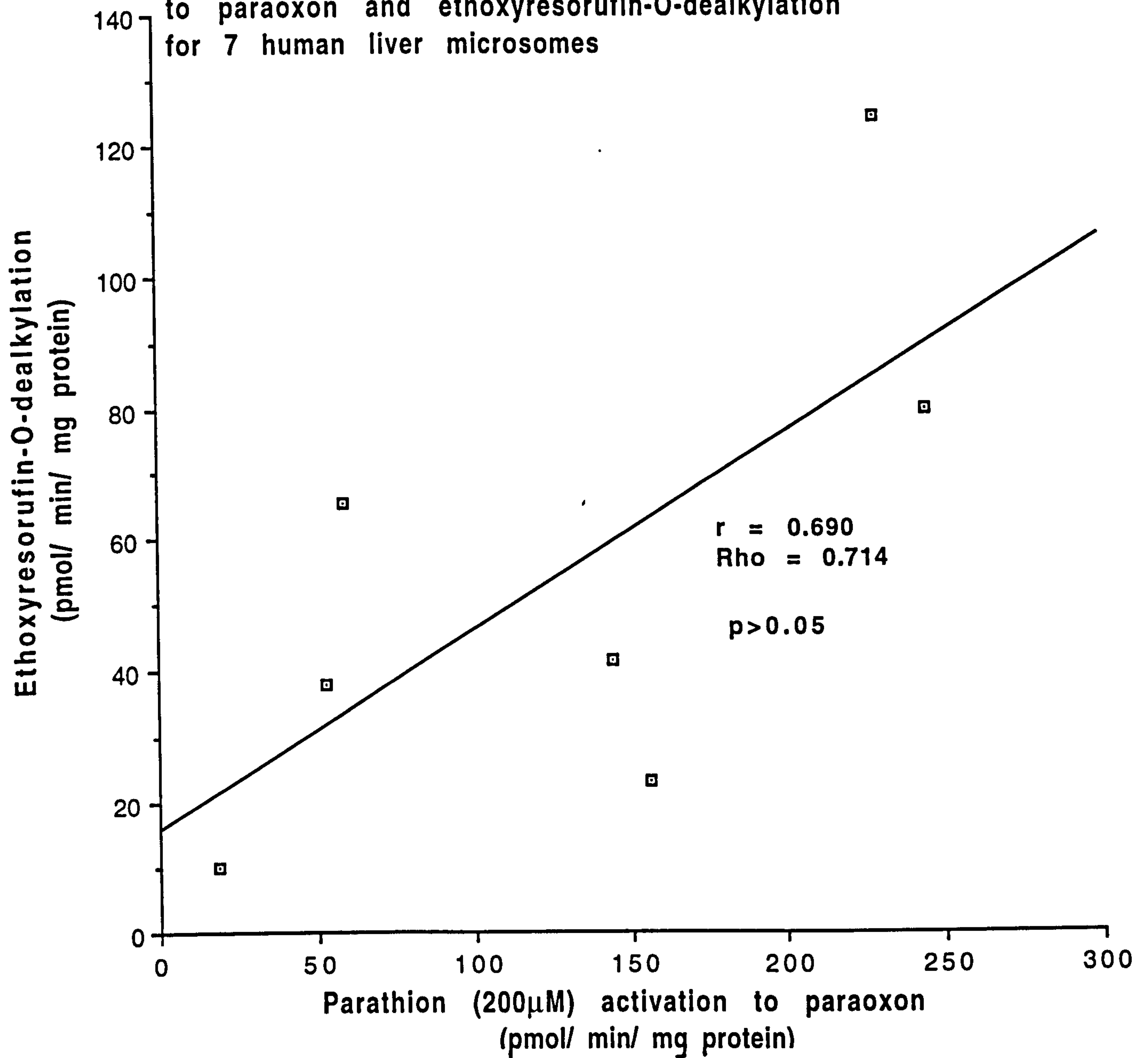


Figure 12.16

Correlation between the activation of parathion (200 μ M)
to paraoxon and pentoxoresorufin-O-dealkylation

for 7 human liver microsomes

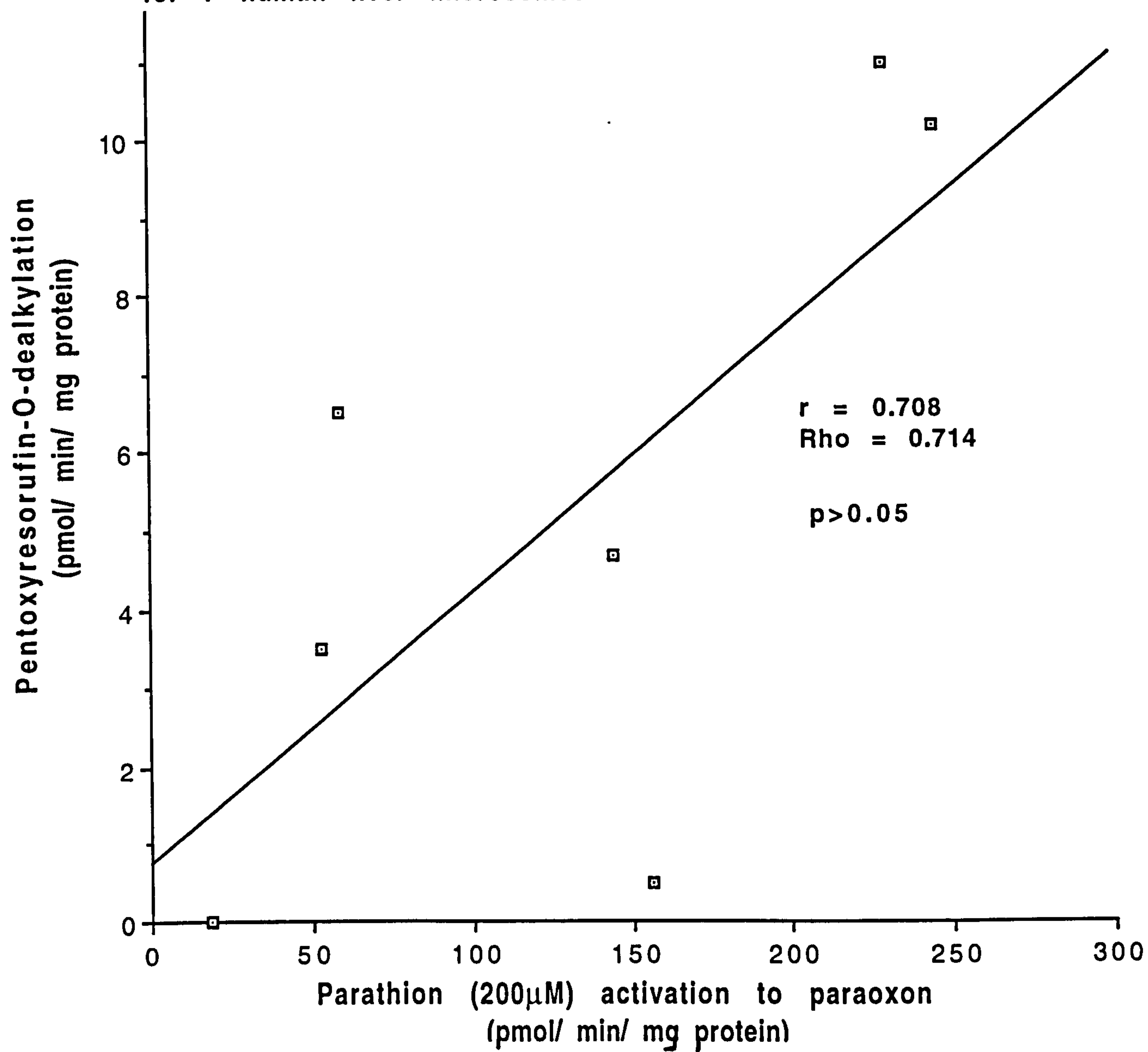


Figure 12.17
Correlation between nifedipine oxidation and
pentoxyresorufin-O-dealkylation for
7 human liver microsomes

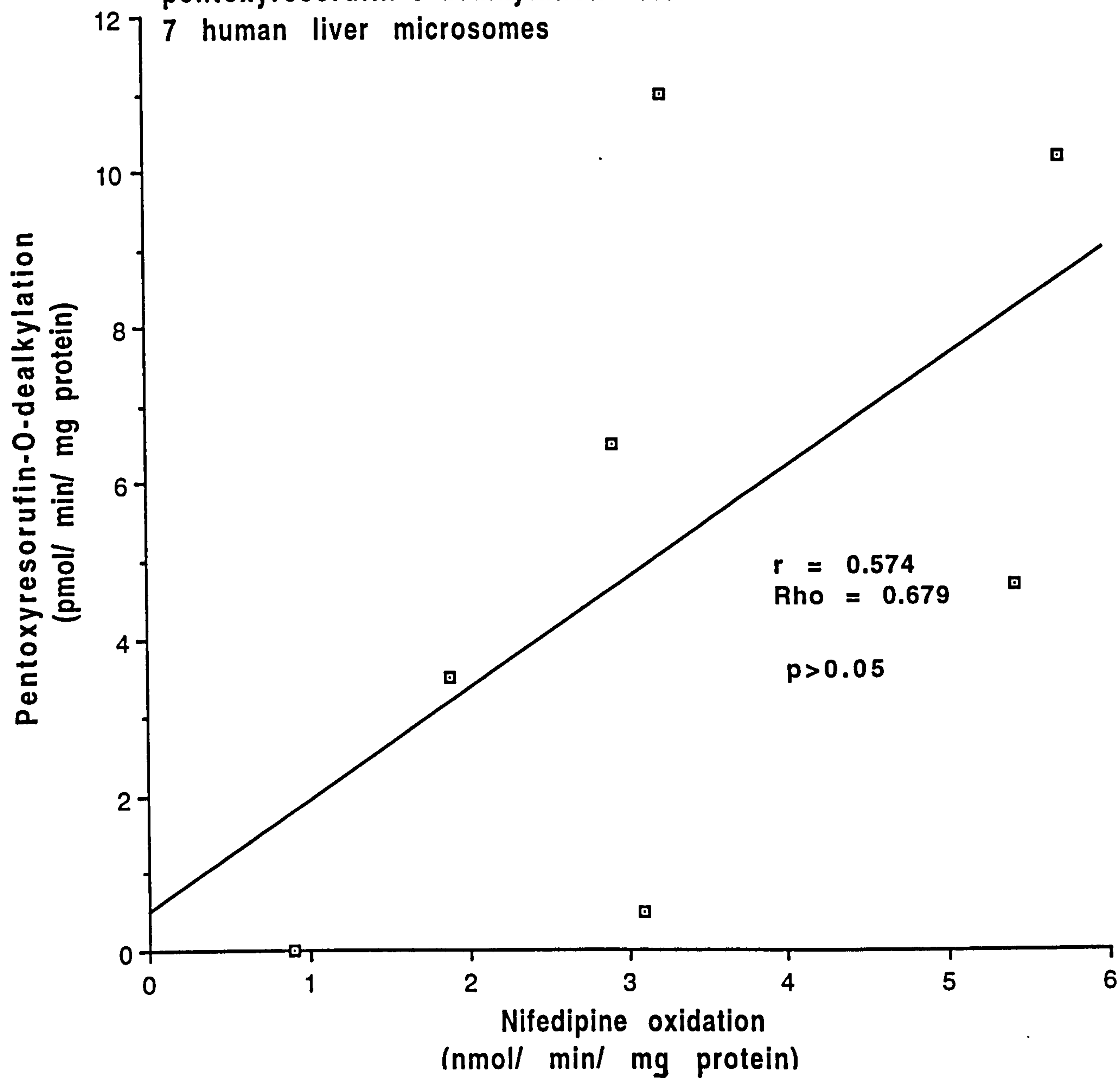


Table 12.8 Correlations between enzyme activities and paraoxon and *p*-nitrophenol formation from parathion (200μM).

Enzyme reaction	n	Correlation with paraoxon formation	Correlation with <i>p</i> -nitrophenol formation
Nifedipine oxidation	16	$r = 0.582^*$ $Rho = 0.657^{**}$	$r = 0.288$ $Rho = 0.369$
Ethoxyresorufin-O-dealkylation	7	$r = 0.690$ $Rho = 0.714$	$r = 0.253$ $Rho = 0.286$
Pentoxyresorufin-O-dealkylation	7	$r = 0.708$ $Rho = 0.714$	$r = 0.258$ $Rho = 0.286$
<i>p</i> -Nitrophenol hydroxylation	7	$r = 0.513$ $Rho = 0.393$	$r = 0.302$ $Rho = -0.107$
Paraoxon hydrolysis	7	$r = -0.057$ $Rho = -0.036$	$r = -0.510$ $Rho = 0.321$
Phenylvalerate hydrolysis	7	$r = -0.142$ $Rho = -0.179$	$r = -0.137$ $Rho = 0.00$
Glutathione conjugation	7	$r = -0.057$ $Rho = -0.036$	$r = -0.510$ $Rho = -0.321$

* $p < 0.02$

** $p < 0.05$

Rho = Spearman rank coefficient

Correlations for parathion (200μM) activation with metabolism by the other substrates was determined for seven of the human liver microsomal preparations (table 12.8). Correlations with ethoxyresorufin-O-dealkylation and pentoxyresorufin-O-dealkylation just failed to achieve statistical significance ($p > 0.05$) (figures 12.15 and 12.16). The correlations were not significant with *p*-nitrophenol hydroxylation, paraoxon hydrolysis, phenylvalerate hydrolysis or glutathione conjugation. Correlation between nifedipine oxidation and pentoxyresorufin-O-dealkylation ($r = 0.574$ and Spearman rank correlation coefficient = 0.679) just failed to achieve statistical significance ($p > 0.05$) (figure 12.17).

12.3.7 Studies with P450-expressed microsomes

The microsomal preparations from human P450-expressed cell lines activated parathion to paraoxon at different rates. Data are presented in table 12.9.

Table 12.9 Metabolic activity of microsomes prepared from human P450-expressed cell lines. Values are the mean of two determinations.

Microsomes expressing	Substrate	Enzyme activity		
		Paraoxon formed ^a	<i>p</i> - nitrophenol formed ^b	M1 formed ^c
CYP3A4	Parathion	11.7	17.8	--
CYP3A5	Parathion	8.8	n.d.	--
CYP1A1	Parathion	4.1	n.d.	--
CYP2B6	Parathion	4.3	n.d.	--
CYP2E1	Parathion	n.d.	n.d.	--
CYP2C8	Parathion	2.4	n.d.	--
CYP2C9-Arg ₁₄₄	Parathion	n.d.	n.d.	--
CYP3A4	Nifedipine	--	--	65.0
CYP3A5	Nifedipine	--	--	28.9

^a pmol paraoxon formed hr⁻¹ pmol P450⁻¹ from 200μM parathion

^b pmol *p*nitrophenol formed hr⁻¹ pmol P450⁻¹ from 200μM parathion

^c pmol M1 formed hr⁻¹ pmol P450⁻¹ from 100μM nifedipine

-- not determined

n.d. below the limit of detection (35pmol paraoxon and 10pmol *p*nitrophenol)

P450s 3A4 and 3A5 were the most efficient at producing paraoxon (11.7 and 8.8 pmol/ hr/ pmol P450, respectively), although P450s 1A1, 2B6 and 2C8 also catalysed the reaction. Formation of paraoxon by P450s 2E1 and 2C9

was below the analytical limit of detection. *p*-Nitrophenol formation was only detectable with P450 3A4, at a rate of 17.8 pmol/ hr/ pmol P450. As expected, nifedipine was metabolised by P450s 3A4 and 3A5 and this was approximately five-fold more rapid than parathion activation.

12.4 Discussion

Initial studies with human liver microsomes defined interindividual variations in the cytochromes P450 and esterases which may be involved in the metabolism of parathion and found them to be comparable with literature reports.

Human liver microsomes were also shown to catalyse NADPH-dependent formation of paraoxon from parathion with nine- and sixteen-fold ranges in activity, depending on substrate concentration. However, the range for *p*-nitrophenol formation was only twofold, regardless of substrate concentration. The kinetic data indicated similar K_m 's for paraoxon and *p*-nitrophenol formation, and both reactions appeared to be monophasic in nature.

It seems reasonable to suggest that a high paraoxon/ *p*-nitrophenol ratio may give an indication of increased susceptibility to parathion toxicity since these individuals appear to have a greater capacity to activate the phosphorothioate. However, it also seems possible that *p*-nitrophenol was further metabolised, although it is unlikely that this was exclusively mediated by CYP2E1 since the *p*-nitrophenol levels (approximately 5 μ M) attained were below the K_m for this isoform, 26.3 μ M (Tassaneeyakul *et al*, 1993). A study by Zerilli *et al* (1997) demonstrated that CYP3A made a significant contribution to *p*-nitrophenol metabolism, especially in

individuals with high levels of this isoform, and it is possible that CYP3A contributed to the further metabolism of *p*-nitrophenol in this study.

The studies with naringenin, quercetin and ketoconazole at high and low substrate concentrations suggested that the CYP3A subfamily was involved in parathion activation by human liver microsomes. Troleandomycin was a weak inhibitor of the reaction. Other workers have also found troleandomycin to be an ineffective inhibitor of the metabolism of several CYP3A substrates such as dapsone (Fleming *et al*, 1992) and taxol (Kumar *et al*, 1993). The exact reasons for this are not known but it has been suggested (Koley *et al*, 1995) that P450 3A is prone to allosteric effects, moreover troleandomycin has been shown to be very slow-acting in its role as a suicide inhibitor (Chang *et al*, 1994). The lesser inhibitions observed with α -naphthoflavone and metyrapone implied some involvement of P450s 1A and 2B6, respectively.

Inhibition with sulphaphenazole was variable in the three microsomal preparations examined and highlights the problems of investigating small sample numbers. Interestingly, one liver, characterised as having very low CYP3A (nifedipine oxidase; 0.055 nmol/ min/ mg protein) and high CYP2C activity (Prof. G Hawksworth, personal communication) demonstrated a high capacity to activate parathion (112 pmol/ min/ mg protein). This data implies that CYP2C may be involved in parathion activation in livers low in CYP3A. Unfortunately, the human liver panel was not characterised for CYP2C activity and therefore it was not possible to assess a correlation between parathion metabolism and this subfamily. Previous studies (Butler and Murray, 1997), however, demonstrated a poor correlation between tolbutamide hydroxylation (a CYP2C9-mediated

reaction) and parathion metabolism for eleven human liver microsomal preparations.

The inhibition data were in agreement with those of Butler and Murray (1997) who also observed inhibition of parathion metabolism by CYP3A inhibitors. They showed about 60% inhibition with ketoconazole (25 μ M), but little effect with troleandomycin (500 μ M) or α -naphthoflavone (100 μ M). These researchers suggested that CYP1A2, as well as CYP3A4, was involved in parathion metabolism since these isoforms were most susceptible to loss of holo-P450 following preincubation with parathion in the presence of NADPH.

Incubations with human P450-expressed microsomes showed that CYPs 3A4 and 3A5 were capable of activating parathion and oxidising nifedipine and that 3A4 was two-fold more active than 3A5 in metabolising both substrates. P450s 1A1, 2B6 and 2C8 were also capable of activating parathion, albeit less efficiently than P450s 3A4 and 3A5, but P450 3A4 alone had the capacity to also detoxify the phosphorothioate to *p*-nitrophenol. O'Shaughnessy and Sultatos (1995) demonstrated the capacity of human P450 2B6 expressed microsomes to form both paraoxon and *p*-nitrophenol from parathion and a very low rate of formation of both metabolites by P450 2E1 although activities were not based on P450 content.

Using a conversion value of 250pmol CYP3A/ mg microsomal protein (Guengerich and Turvy, 1990), the rate of formation of paraoxon by the panel of human liver microsomes, 75-1241 pmol/ min/ nmol 3A, was similar to that of the CYP3A4-expressed cells (195 pmol/ min/ nmol 3A4). Moreover, the ratio of activation to detoxification of parathion by CYP3A4

expressed microsomes was 0.66 and compared favourably to that calculated for the panel of human livers.

The study showed a positive correlation between parathion (200 μ M) activation to paraoxon and nifedipine oxidation, indicating the involvement of CYP3A in the reaction. This corroborates another study of human liver microsomes in which a significant correlation between parathion metabolism and testosterone 6 β -hydroxylation (a CYP3A reaction) was disclosed (Butler and Murray, 1997). In the study with the smaller group of liver microsomes the correlations between parathion activation and ethoxyresorufin-O-dealkylation and pentoxyresorufin-O-dealkylation just failed to achieve statistical significance. However, the relationship with pentoxyresorufin-O-dealkylation may have been confounded by the non-specificity of this reaction since pentoxyresorufin has been shown to be a substrate for CYP3A4, as well as CYP2B6, in human liver microsomes (Waziers *et al*, 1990). Indeed, the correlation between nifedipine oxidation and pentoxyresorufin-O-dealkylation just failed to achieve statistical significance.

Hydrolysis of paraoxon *in vitro* has been shown to be mediated by the calcium-dependent A-esterase (paraoxonase) of human plasma (Mackness, 1989) and liver (Gil *et al*, 1993) at high substrate concentrations (1-5mM). However, recent studies (Chambers *et al*, 1994) with rat liver microsomes have suggested that paraoxonase is not functionally important *in vitro* or *in vivo* at toxicologically relevant nM concentrations of paraoxon, since these are well below the K_m of the enzyme, 0.4mM (Gil *et al*, 1993).

Experiments were therefore established to determine the role of paraoxonase in the formation of *p*-nitrophenol from paraoxon at the

levels (1-3 μ M) achieved in incubations with 20 μ M and 200 μ M parathion. *p*-Nitrophenol was not detected in incubations with paraoxon (1-3 μ M) alone or including 5mM EDTA, a paraoxonase inhibitor. Conversely, incubations with a saturating concentration of paraoxon (1mM) and EDTA produced 72% of control *p*-nitrophenol levels. Moreover, there was a very poor correlation between parathion (200 μ M) activation and paraoxonase (1mM paraoxon) activity for the panel of human liver microsomes. There was also a very poor correlation with phenylvalerate hydrolase activity, perhaps suggesting that these esterases do not play an important role in the detoxification of paraoxon by hydrolysis, although their role in removing oxons by covalent binding could not be assessed. An activity range was also determined for glutathione-S-transferases, although these enzymes are not now believed to be important in parathion detoxification, *in vivo* (Sultatos, 1992; Chambers *et al*, 1994). This observation seems to be supported by the poor correlation obtained for parathion metabolism and glutathione-S-transferase activity, albeit for a small population of human livers.

These experiments indicated that paraoxonase was not functionally important at the low levels of paraoxon formed from parathion, but that the enzyme was involved in the reaction at saturating substrate concentrations. However, it is possible that the paraoxon levels measured *in vitro* were reduced by binding to carboxylesterases, although there was no difference between levels of paraoxon measured in the presence and absence of microsomal protein.

Several of these studies with human liver microsomes tend to support the hypothesis first proposed by Nakatsugawa and Dahm (1967) that both paraoxon and *p*-nitrophenol are produced from parathion by way of a

“common intermediate”. P450 was proposed to donate a singlet oxygen to the sulphur moiety of parathion to produce an unstable phosphooxythiran intermediate which then spontaneously rearranged to produce the two metabolites (figure 3.6). An alternative pathway for metabolite formation would be parathion--> paraoxon--> *p*-nitrophenol.

Firstly, these studies demonstrated similar Kms for paraoxon and *p*-nitrophenol formation. It is possible that *p*-nitrophenol followed P450-mediated dearylation of parathion, but this seems unlikely since it would have had to be carried out with the same affinity as parathion desulphuration (to form paraoxon). Also, the inhibition studies showed that *p*-nitrophenol and paraoxon were affected in parallel by the compounds investigated. Moreover, it seems more likely that *p*-nitrophenol was produced directly from a common intermediate since A-esterases did not appear to be involved in the detoxification of paraoxon at biologically relevant concentrations of parathion.

Elucidation of the “common intermediate” theory has important implications for parathion toxicity. Confirmation of the theory would suggest that a P450-mediated reaction is responsible for both the activation and detoxification of this phosphorothioate, at least following low level exposures, although other pathways will be involved in further metabolism of the metabolites, eg carboxylesterase-mediated covalent binding of the oxon.

Considered as a whole, CYP3A4/5 is strongly implicated as the principle catalyst of parathion metabolism, although the possibility exists that CYP1A1/2 may make a contribution, particularly following induction. Participation of CYPs 2B6 and 2C8 cannot be ruled out. Unfortunately, due

to availability of microsomes, the P450-expressed microsome studies were not extended to include assessment of kinetic parameters. Determination of a K_m for parathion metabolism by individual P450s would allow an evaluation of their relative importance *in vivo*. If the K_m values are similar, then the contribution to metabolism by the more abundant form(s) will predominate in the liver. Minor isoforms, eg CYP2B6, will only contribute significantly to metabolism if this is mediated with relatively high affinity. In contrast to CYP3A, hepatic levels for CYPs 1A1/2 and 2B6 are only about 13% and 1%, respectively (Shimada *et al*, 1994), and therefore, referring to their relative liver content, and if affinity is similar between the isoforms, CYP3A would be the major P450 subfamily involved in parathion metabolism *in vivo*. Further studies are needed to confirm or refute the participation of CYP2C8 in the reaction, but the CYP2C subfamily is recognised as a major P450, being 20% abundant (Shimada *et al*, 1994).

SECTION IV:
GENERAL DISCUSSION

Chapter 13

Chapter 13 General discussion

13.1 General discussion

This research determined interindividual variations in the capacity of human liver microsomes to metabolise parathion and investigated the enzyme pathways involved in its biotransformation to paraoxon and *p*-nitrophenol. The animal component of the research intended to complement the data obtained from the limited supply of human liver. In many ways this aim was achieved, but the study also highlighted some of the many problems associated with extrapolation of animal data to man.

The levels of individual P450 isoforms, and indeed the expression of distinct isoforms, are different in the rat compared to man and this led to differences in the metabolism of alleged probe substrates between the species. Notably, the induction and inhibition studies suggested that nifedipine was not specific for CYP3A activity in the rat. Other substrates would perhaps have been better. For example, it has been shown that individual pathways of testosterone oxidation are specific for many rat P450 isoforms (Sonderfan *et al*, 1987). Sonderfan *et al* (1987) demonstrated that 2 α -, 7 α and 16 β -hydroxylation of testosterone accurately reflects the levels of CYPs 2C11, 2A1 and 2B1/2, respectively, while the 2 β -, 6 β - and 15 β -hydroxylation pathways collectively reflect the levels of CYPs 3A1 and 3A2.

In addition, these differences influenced the selectivity of the inhibitors used in the study. For example, sulphaphenazole had no effect on parathion metabolism in the rat, even though the inhibition studies suggested that CYP3A was not the sole isoform responsible for catalysis of

this phosphorothioate and that CYP2C11 was probably also involved. Sulphaphenazole inhibited parathion metabolism in the human liver microsomes investigated, albeit variably. The variability in inhibitory potential of sulphaphenazole between species has also been demonstrated by Veronese *et al* (1990) and others (eg Eagling *et al*, 1998) in their comparative studies of CYP2C-mediated reactions in human, rat and rabbit.

It is well recognised that humans often differ from rodents in their responsiveness to P450 inducers. For example, rifampicin is a CYP3A inducer in humans but not mice or rats (Pichard *et al*, 1990); PCN and mephenytoin are CYP3A inducers in rats but do not appear to induce these P450 isoforms in humans (Parkinson, 1996); barbiturates, such as phenobarbital, are well known powerful inducers of cytochromes P450 2B1/2 (as well as other enzyme systems) in rat liver, whereas drug interactions with phenobarbital in man are thought to stem largely from induction of the CYP3A and CYP2C subfamilies (Parkinson, 1996). As well as the recognised species differences in response to inducers, interpretation of the induction data in this study was confounded by the fact that induction by a particular compound was not specific to an individual P450 isoform. For example, dexamethasone increased ethoxyresorufin-O-dealkylase, pentoxyresorufin-O-dealkylase and *p*-nitrophenol hydroxylase activities, as well as nifedipine oxidation.

In terms of parathion's acute toxic potential, several researchers have shown that phenobarbital pre-treatment in rodents protects against the adverse effects of this, and other, phosphorothioates (Alary and Brodeur, 1969; Sultatos, 1986; Chambers *et al*, 1994). The literature suggests, however, that the action of phenobarbital on parathion toxicity cannot be

attributed exclusively to alterations in P450-mediated oxidative desulphuration since phenobarbital has also been shown to antagonise the toxicity of paraoxon (Vitarius *et al*, 1995a). The present rat studies corroborated the well known observation that exposure of animals to phenobarbital substantially increases hepatic P450 levels, and the data showed that both CYP2B activity and paraoxon formation from parathion were increased by this pre-treatment.

The phenobarbital-treated liver was enlarged, had a greatly increased level of microsomal proteins, which would include microsomal carboxylesterases (Chambers *et al*, 1994), and an increased capacity for carboxylesterase-mediated hydrolysis. This would infer a substantially increased hepatic capacity for detoxification of parathion *in vivo*, despite the fact that activation would also be increased. All of these detoxification pathways probably contribute to the attenuation of parathion toxicity observed following phenobarbital pre-treatment. However, covalent binding of the oxon to carboxylesterases would possibly be of greatest importance in limiting the amount of toxin leaving the liver and entering the systemic circulation, particularly since phenobarbital decreased hepatic paraoxonase activity, an observation also noted by others (Vitarius *et al*, 1995). Sultatos (1986) also demonstrated that, despite increased paraoxon production in the phenobarbital-treated mouse, there was no increase in paraoxon exiting the perfused liver, while there were increases in detoxified metabolites.

Ethanol has also been shown to antagonise the acute toxicity of parathion (O'Shaughnessy *et al*, 1995). O'Shaughnessy *et al* (1995) hypothesised that chronic ethanol exposure leads to an increased rate of oxygen consumption in the rat which may result in reduced availability to the

hepatic P450s, and compromised parathion activation. The present study showed no increase in paraoxon production from parathion compared to control and increased CYP2E1 activity, although this isoform did not appear to be involved in parathion metabolism. However, protein recovery (but not P450 recovery) was substantially increased, although this was rather counter-balanced by a decrease in liver weight. These data again emphasise the importance of covalent binding to the carboxylesterases in attenuation of toxicity, although ethanol pre-treatment did also increase paraoxonase activity.

The animal component of the study therefore emphasised the necessity for preliminary "in house" validation of the substrates and inhibitors of interest. Failure to do so may lead to the incorrect assignment of P450 involvement if animal studies alone are used as a model for human drug metabolism. Even though caution should be exercised when extrapolating animal data to the human situation, the studies indicated that CYP3A played a role in parathion metabolism in control rat microsomes, but it was not the only P450 isoform involved. It is possible that CYP2C also participated in the reaction, but the present data could not support this hypothesis. Cytochromes P450 3A, 1A and possibly 2B were implicated in the induced animal.

The animal component of this research, and other animal studies, have shown that P450-dependent activation of parathion plays a critical role in controlling the extent of acetylcholinesterase inhibition following exposure (Chambers and Chambers, 1990; Sawyer *et al*, 1992). This will, however, be a balance between the various detoxification pathways outlined in the previous studies. It follows that factors which alter the metabolism of such phosphorothioates could potentially alter their acute

toxicity. Unlike inbred animals, however, human individuals will vary greatly in their toxic response to phosphorothioates and this variability was investigated in the second part of the thesis.

The human section of this research concentrated on studies with human liver microsomal preparations and commercially available human P450-expressed microsomes. Taken together, these data indicated the involvement of CYPs 3A4/5, 1A1/2 and possibly 2C8 in parathion metabolism in man, although CYP3A4/5 was strongly implicated as the principle catalyst. However, CYP1A could also be of importance *in vivo*, particularly in individuals with high levels of this isoform, for example following induction, and if the reaction was mediated with relatively high affinity. This is particularly important since CYP1A1 (and to some extent CYP1A2) is well known for its potent inducibility by various aromatic hydrocarbons, and an apparent polymorphism with respect to inducibility of CYP1A1 by 3-methylcholanthrene has been described (Kellerman *et al*, 1973). Further studies would be necessary to assess participation of CYP2C8 in the metabolism of parathion and to determine the relative affinities of all the P450s involved in the reaction.

Without exception, the levels and activity of each of the P450 isoforms will vary in the individuals studied, due to the many environmental and/or genetic factors described in the earlier chapters. In particular, this study showed a typically wide range in nifedipine oxidase activity which reflected the wide (>tenfold) interindividual variation observed in levels of hepatic CYP3A protein (Shimada *et al*, 1989). The wide range in nifedipine oxidase activity may be the result of several factors, including variability in glucocorticoid levels (which induce CYP3A4) and CYP3A5 expression, although whether there is a genuine polymorphism in

CYP3A4 remains to be established. Although one individual in this study had very low CYP3A activity determined phenotypically, there is no evidence for the existence of any individual who completely lacks CYP3A activity.

In the present study paraoxon formation from parathion by human liver microsomes *in vitro* was mediated with nine- and sixteen-fold ranges in activity, depending on substrate concentration. It is attractive to suggest that those individuals shown to have a greater capacity to produce paraoxon *in vitro*, which paralleled higher activities for CYP3A4/5 and/or CYP1A, may be more susceptible to parathion toxicity *in vivo*. This is obviously an over-simplification and takes no account of the many modifying factors described in the earlier chapters.

These studies were conducted using *in vitro* systems, but the situation would be further complicated *in vivo* because of variable expression of the phase 2 enzymes, which were not investigated in this thesis. Both the UDP glucuronosyltransferases and sulphotransferases could be expected to participate in the further metabolism of *p*-nitrophenol, *in vivo*. Classically, sulphotransferase is a detoxification pathway of high affinity but low capacity, whereas glucuronidation has a lower affinity but a higher capacity. In terms of K_m , it has been proposed that at concentrations below $10\mu\text{M}$ *p*-nitrophenol will be primarily sulphated, while glucuronidation will predominate above $10\mu\text{M}$ (Sultatos and Minor, 1985). The considerable variation in sulphotransferase activity between individuals, particularly P-PST activity which has been shown to vary due to several allelic variants in man, may be of importance for parathion toxicity *in vivo* (Coughtrie, 1996).

An accurate estimation of the concentration of parathion that may be encountered in man *in vivo* is difficult to establish. However, it has been suggested that the minimum acute lethal dose of parathion in adults could be as little as 4mg/ kg (Gosselin *et al*, 1984). This value relates to a study of mice (Sultatos *et al*, 1985) in which the hepatic portal blood concentration of parathion reached a peak of 9 μ M thirty minutes after a near lethal dose (13mg/ kg). This transiently high level of parathion was decreased to 2.3 μ M forty-five minutes post dose.

There is a paucity of information in the literature regarding the possible body burden of parathion following occupational exposure. This will obviously be far lower than the suggested minimum acute lethal dose, although exposure will probably be multiple in nature. The literature indicates that occupational exposure to phosphorothioates will be mainly dermal and perhaps also via the pulmonary and/ or oral routes in some instances, such as following use of aerosols in confined spaces, splashing during the sheep-dipping process etc., if insufficient protective clothing is employed. Studies carried out by Niven *et al* (1993) and Nutley and Cocker (1993) have indicated a low internalised dose of phosphorothioates as measured by blood enzymes and urinary metabolite levels. Such studies have also suggested that there is widespread lack of compliance with regard to the guidelines for worker protection during occupational use of phosphorothioates, but measurements are not always taken during the times of non-compliance.

Following low-level, multiple exposures to an animal by the oral route, the carboxylesterases could be expected to covalently bind much of the hepatically generated oxon, and it seems unlikely that appreciable amounts of toxin would reach target tissues. Occupational exposure

following the dermal route, however, would not be subject to first pass metabolism in the liver, and therefore it is possible that some unaltered phosphorothioate (and oxon) may reach the brain. The sensitivity of the brain to toxic insult and its low margin of safety may mean that "little" may be "enough" to produce adverse effects. An *in vitro* study by Sorrano and Sultatos (1992) has shown parathion to be metabolised to *p*-nitrophenol by the brain although no paraoxon was detected, presumably because it was covalently bound to the tissue carboxylesterases and therefore not extractable.

Several studies by Chambers and co-workers (Chambers *et al*, 1989; Chambers *et al*, 1991; Chambers, 1992) with rats have suggested that the ability of the brain to locally activate phosphorothioate pesticides greatly influences manifest acute toxicity in this animal. They base this theory on studies with six phosphorothioates, including parathion, and demonstrated that those pesticides that were most toxic in the whole animal were activated by brain to the greatest extent. Chambers also speculated that while brain activation of phosphorothioates is probably of little consequence in severe acute poisoning due to the large amounts of hepatically generated oxon, it may be of significance following single or multiple low dose exposures such as experienced by man in the workplace situation.

The cytochromes P450 of the lung have also been shown to be capable of activating parathion, with a K_m of 7.9 μM , which is similar to that determined in this thesis for hepatic metabolism (Norman and Neal, 1976; Lessire *et al*, 1996). Several researchers have shown that the lung metabolises parathion very rapidly (Law *et al*, 1974; Norman and Neal, 1976; Lessire *et al*, 1996). For example, Law *et al* (1974) demonstrated that it

took less than three minutes for paraoxon to appear in a recirculating perfusion system where rabbit lungs were dosed with parathion. Therefore, even relatively low occupational levels of parathion which may enter the lung by inhalation could be rapidly activated to the oxon and would then be available to exert anti-cholinesterase effects at this target tissue. The lung appears to be particularly important in this respect since the cause of death in acute poisoning is respiratory failure resulting from paralysis of the muscles of respiration, bronchioconstriction, accumulation of fluid in the lungs, as well as central nervous system effects (Neal, 1972).

In conclusion, this research has determined marked variability in capacity to metabolise parathion by human liver microsomal preparations, *in vitro*. There is potential for the alteration of parathion disposition by co-administration of drugs or food products which are either inducers or competing substrates of the CYP3A and CYP1A subfamilies. Inducers would include the drugs dexamethasone and phenobarbital (CYP3A), and cigarette smoke condensate (CYP1A1) while natural flavonoids such as naringenin and quercetin and the drugs nifedipine and erythromycin could be expected to compete for the CYP3A active site.

These data suggest that a higher hepatic capacity to activate parathion may influence the toxicity of this phosphorothioate, particularly following a high acute dose, but the results should be applied with caution. Additional toxicological studies using low level multiple doses of parathion paralleled by an investigation of human capacity to metabolise such phosphorothioates, in both hepatic and extrahepatic tissues, are necessary to further elucidate interindividual differences in toxic outcome following exposure.

13.2 Future studies

This research has suggested several areas for future study. Firstly, the P450-expressed microsome studies should be extended to include determination of individual K_m 's for the isoforms proposed to participate in parathion metabolism in man, that is CYPs 3A4, 3A5, 1A1, 1A2 and possibly CYPs 2C8 and 2B6. Using a pure enzyme source in this way would determine the affinity of each isoform in the reaction. The information obtained could then be improved by taking into account the extent to which the individual isoforms are expressed in human liver microsomes (Guengerich and Turvy, 1990). This would then allow for assessment of the relative contribution of the isoforms to parathion metabolism *in vivo*.

Preliminary studies in the department using rat lung microsomes confirmed the observation of others (Norman and Neal, 1976) that this tissue is capable of activating and detoxifying parathion, at about 20% of the rate produced by liver microsomes (13.0 ± 1.5 and 18.2 ± 3.8 pmoles/min/mg protein for paraoxon and *p*-nitrophenol formation, respectively). The reaction was entirely dependent on NADPH and preliminary inhibition studies suggested participation of CYP2B, although it was not the only isoform involved. Several samples of human lung have yet to be studied. Studies with rat skin microsomes suggested that parathion metabolism in this tissue was below the limit of detection using the method described previously. One experiment using human brain microsomes showed nifedipine oxidation activity to also be below the limit of detection. In view of these observations, further studies should include development of more sensitive techniques to ascertain parathion metabolism in these extrahepatic tissues and to determine involvement of the individual P450 isoforms. Other, more occupationally relevant

phosphorothioates, such as diazinon, chlorpyrifos and propetamphos, should then be investigated using similar techniques in order to predict individual susceptibility to the toxic effects of these pesticides.

An animal component should be carried out in parallel to determine blood and target tissue levels of phosphorothioate/ oxon with markers of effect (target/ erythrocyte acetylcholinesterase inhibition and urinary metabolites) following low-level, multiple exposure. This could be studied alongside experiments to assess hepatic and brain/ lung capacity to metabolise the phosphorothioate *in vitro*.

SECTION V:
APPENDICES and REFERENCES

Chapter 14

Appendix I

This appendix relates to rat inhibition results described in chapter 10.

Table 1 Inhibition of M1 formation from nifedipine (5μM and 100μM) by rat liver microsomal preparations (n=2, except where indicated). Values are percent of control activity (mean ± sem).

Inhibitor (conc.)	Nifedipine concentration	
	5μM	100μM
None (control)	0.245 (nmol/ min/ mg protein)	1.69 ± 0.10* (nmol/ min/ mg protein)
Naringenin (100μM)	--	35.3 ± 9.0*
TAO (100μM)	--	104.7 ± 22.0*
KCZ (100μM)	--	n.d.
Quercetin (100μM)	--	n.d.
Quinidine (100μM)	--	33.0 ± 8.0*
Naringenin (40μM)	34.0	28.0
TAO (40μM)	44.0	50.0
KCZ (40μM)	24.0	37.0

* n=3

-- not determined

n.d. = below the limit of detection (0.01 nmol M1/ incubation)

TAO = troleandomycin

KCZ = ketoconazole

Table 2 Inhibition of paraoxon and *p*-nitrophenol formation from parathion (200μM) by rat liver microsomal preparations (n=3). Values are percent of control activity (mean ± sem).

Inhibitor (1mM)	Paraoxon formation (% control)	<i>p</i> -Nitrophenol formation (% control)
None (control)	248 ± 10 (pmol/ min/ mg protein)	227 ± 18 (pmol/ min/ mg protein)
Naringenin	52.3 ± 6.5	52.7 ± 3.9
Ketoconazole	81.5 ± 2.1	70.8 ± 3.4
Metyrapone	97.0 ± 3.1	83.7 ± 0.9
α-Naphthoflavone	83.0 ± 11.7	87.8 ± 5.1
Diethyldithiocarbamate	124.0 ± 6.6	121.0 ± 4.1
Tolbutamide	115.0 ± 11.3	98.8 ± 9.6

Table 3 Inhibition of paraoxon and *p*-nitrophenol formation from parathion (200μM) by rat liver microsomal preparations (n=2). Values are percent of control activity (mean).

Inhibitor (100μM)	Paraoxon formation (% control)	<i>p</i> -Nitrophenol formation (% control)
None (control)	262 ± 10 (pmol/ min/ mg protein)	232 ± 18 (pmol/ min/ mg protein)
Naringenin	66.6	58.4
Ketoconazole	80.0	89.0
Metyrapone	113.0	107.0
α-Naphthoflavone	107.4	97.0
Diethyldithiocarbamate	91.3	99.7
Tolbutamide	111.5	111.5
Troleandomycin	112.5	129.5
Quercetin	85.3	77.0
Quinidine	100.5	117.5

Table 4 Inhibition of paraoxon and *p*-nitrophenol formation from parathion (20μM) by rat liver microsomal preparations (n=2). Values are percent of control activity (mean).

Inhibitor (100μM)	Paraoxon formation (% control)	<i>p</i> -Nitrophenol formation (% control)
None (control)	210 ± 10 (pmol/ min/ mg protein)	212 ± 15 (pmol/ min/ mg protein)
Naringenin	56.2	57.3
Ketoconazole	80.7	78.0
Metyrapone	98.0	106.3
α-Naphthoflavone	89.8	90.5
Diethyldithiocarbamate	70.7	97.1
Tolbutamide	98.6	103.9
Troleandomycin	128.0	111.5
Quercetin	78.1	74.8
Quinidine	122.5	98.8

Appendix II

This appendix relates to studies described in chapter 12.2.

Table 1 Microsomal protein recovery from approximately 1g of liver for 16 human livers.

Laboratory number	Lowry protein (mg / ml)	Protein recovery (mg protein/ g liver)
M1	6.0	19.6
M2	4.5	19.1
M3	10.2	20.3
M4	4.0	15.6
M5	3.9	12.4
M6	5.8	13.4
M7	3.5	17.6
M8	4.7	8.5
M9	8.0	15.1
M10	6.6	10.3
M11	6.4	11.5
M12	6.7	12.9
M13	5.7	10.7
M14	7.6	12.6
M15	7.5	15.0
M16	9.6	17.2
Mean ± sem	6.3 ± 0.49	14.5 ± 0.89

Appendix III

This appendix relates to results described in chapter 12.3.

Table 1 Nifedipine oxidase activities of human liver microsomes. Results are the mean \pm sem and range with median in parenthesis for n=16 samples. The substrate concentration was 100 μ M.

Laboratory number	ACTIVITY (nmol/ min/ mg protein)
M1	0.902
M2	3.10
M3	5.43
M4	5.72
M5	1.89
M6	3.25
M7	2.92
M8	1.39
M9	2.45
M10	1.19
M11	1.68
M12	0.529
M13	0.055
M14	0.729
M15	3.89
M16	3.51
Mean \pm sem	2.57 \pm 0.41
Range (median)	0.055 - 5.72 (1.72)

Table 2 Ethoxy- and pentoxyresorufin-O-dealkylase activities of human liver microsomal fractions. Results are the mean \pm sem and range with median in parenthesis for n=7 samples. The substrate concentrations were 2 μ M.

Laboratory number	EOR activity (pmol/ min / mg protein)	POR activity (pmol/ min / mg protein)
M1	10.0	n.d.
M2	23.1	0.5
M3	41.7	4.7
M4	79.9	10.2
M5	38.0	3.5
M6	124.4	11.0
M7	65.5	6.5
Mean \pm sem	54.7 \pm 14.7	5.2 \pm 2.0
Range (median)	10.0 - 124.4 (41.7)	0 - 11.0 (4.7)

n.d. = below the analytical limit of detection (0.1pmol/ min)

Table 3 *p*-Nitrophenol hydroxylase activities of human liver microsomal fractions. Results are the mean \pm sem and range with median in parenthesis for n=7 samples. The substrate concentration was 200 μ M.

Laboratory number	ACTIVITY (nmol/ min / mg protein)
M1	0.838
M2	1.32
M3	0.797
M4	1.22
M5	0.772
M6	0.785
M7	0.662
Mean \pm sem	0.913 \pm 0.095
Range (median)	0.662 - 1.32 (0.797)

Table 4 Paraoxonase activity of human liver microsomal fractions. Results are the mean \pm sem and range with median in parenthesis for n=7 samples. The substrate concentration was 1mM.

Laboratory number	ACTIVITY (nmol/ min / mg protein)	ACTIVITY (nmol/ min / g liver)
M1	7.3	141.0
M2	6.6	94.5
M3	16.0	327.0
M4	5.0	59.5
M5	4.6	57.5
M6	9.3	124.0
M7	12.7	166.0
Mean \pm sem	8.8 \pm 1.6	138.5 \pm 34.9
Range (median)	4.6 - 16.0 (7.3)	57.5 - 327.0 (124.0)

Table 5 Phenylvalerate hydrolase activities of human liver microsomal fractions. Results are the mean \pm sem and range with median in parenthesis for n=7 samples. Substrate concentration was 1.4mM.

Laboratory number	ACTIVITY ($\mu\text{mol/ min / mg}$ protein)	ACTIVITY ($\mu\text{mol/ min / g}$ liver)
M1	14.9	291.5
M2	8.42	121.3
M3	9.48	193.3
M4	10.3	120.5
M5	6.34	79.5
M6	12.9	172.2
M7	13.4	124.7
Mean \pm sem	10.8 \pm 1.15	157.6 \pm 26.4
Range (median)	6.34 - 14.9 (10.3)	79.5 - 291.5 (124.7)

Table 6 Glutathione-S-transferase activities of human liver cytosol. Results are the mean \pm sem and range with median in parenthesis for n=7 samples. The substrate concentration was 1mM.

Laboratory number	ACTIVITY (nmol/ min / mg cytosolic protein)	ACTIVITY (μ mol/ min / g liver)
M1	859	72
M2	553	41
M3	568	45
M4	1120	81
M5	1190	72
M6	892	61
M7	978	76
Mean \pm sem	880 \pm 93.6	64 \pm 5.9
Range (median)	553 - 1190 (892)	41 - 81 (72)

Table 7 Parathion activation to paraoxon by human liver microsomes. Results are the mean \pm sem and range with median in parenthesis for n=16 livers. The substrate concentrations were 20 μ M and 200 μ M

Laboratory number	ACTIVITY from 20 μ M parathion (pmol paraoxon/ min/ mg protein)	ACTIVITY from 200 μ M parathion (pmol paraoxon/ min/ mg protein)
M1	49.8	18.7
M2	57.6	156.3
M3	52.8	144.4
M4	80.6	244.5
M5	23.3	53.1
M6	173.6	229.2
M7	70.8	58.7
M8	29.0	110.3
M9	104.5	210.3
M10	64.1	93.0
M11	125.5	175.5
M12	104.9	122.3
M13	107.0	112.0
M14	60.9	117.3
M15	140.7	310.3
M16	199.3	295.7
Mean \pm sem	90.3 \pm 12.5	153.2 \pm 21.3
Range (median)	23.3 - 199.3 (70.8)	18.7 - 310.3 (117.3)

Table 8 Parathion detoxification to *p*-nitrophenol by human liver microsomes. Results are the mean \pm sem and range with median in parenthesis for n=16 samples. The substrate concentrations were 20 μ M and 200 μ M

Laboratory number	ACTIVITY from 20 μ M parathion (pmol <i>p</i> -nitrophenol/ min/ mg protein)	ACTIVITY from 200 μ M parathion (pmol <i>p</i> -nitrophenol/ min/ mg protein)
M1	487.6	515.2
M2	605.7	715.6
M3	321.1	406.2
M4	726.8	746.3
M5	656.9	687.8
M6	644.8	616.9
M7	769.2	778.3
M8	593.2	671.2
M9	446.6	511.4
M10	452.1	494.6
M11	530.7	578.2
M12	510.9	496.6
M13	517.8	538.3
M14	432.0	441.4
M15	508.5	721.5
M16	426.5	499.5
Mean \pm sem	538.0 \pm 26.8	583.2 \pm 26.3
Range (median)	321.1 - 769.2 (513.1)	406.2 - 778.3 (558.1)

Appendix IV

This appendix relates to results described in chapter 12.3.

Table 1 Inhibition of paraoxon formation from parathion (20μM) by two human liver microsomal preparations. Values are percent of control activity (mean of duplicate estimations).

Inhibitor (100μM)	Liver M15	Liver M16	Mean
None (control)	140.7 (pmol/ min/ mg protein)	199.3 (pmol/ min/ mg protein)	100%
Naringenin	41.2	46.4	43.8
Troleandomycin	74.0	90.8	82.4
Quercetin	11.6	24.5	18.1
Ketoconazole	13.0	25.8	19.4
Quinidine	66.5	87.7	77.1

Table 2 Inhibition of paraoxon formation from parathion (20μM) by two human liver microsomal preparations. Values are percent of control activity (mean of duplicate estimations).

Inhibitor (100μM)	Liver M9	Liver M11	Mean
None (control)	104.5 (pmol/ min/ mg protein)	125.5 (pmol/ min/ mg protein)	100%
Metyrapone	84.9	91.5	88.2
α-Naphthoflavone	64.4	63.5	64.0
DEDC	111.2	81.5	96.4
Tolbutamide	98.4	91.5	95.0

DEDC = Diethyldithiocarbamate

Table 3 Inhibition of *p*-nitrophenol formation from parathion (20μM) by two human liver microsomal preparations. Values are percent of control activity (mean of duplicate estimations).

Inhibitor (100μM)	Liver M15	Liver M16	Mean
None (control)	508.5 (pmol/ min/ mg protein)	426.5 (pmol/ min/ mg protein)	100%
Naringenin	64.8	60.4	62.6
Troleandomycin	84.4	71.0	77.7
Quercetin	47.6	43.9	45.8
Ketoconazole	49.2	40.7	45.0
Quinidine	97.4	87.5	92.5

Table 4 Inhibition of *p*-nitrophenol formation from parathion (20μM) by two human liver microsomal preparations. Values are percent of control activity (mean of duplicate estimations).

Inhibitor (100μM)	Liver M9	Liver M11	Mean
None (control)	446.6 (pmol/ min/ mg protein)	530.7 (pmol/ min/ mg protein)	100%
Metyrapone	81.9	111.6	96.8
α-Naphthoflavone	74.0	107.0	90.5
DEDC	101.6	72.6	87.1
Tolbutamide	97.1	103.0	100.1

DEDC = Diethyldithiocarbamate

Table 5 Inhibition of paraoxon formation from parathion (200μM) by two human liver microsomal preparations. Values are percent of control activity (mean of duplicate estimations).

Inhibitor (100μM)	Liver M15	Liver M16	Mean
None (control)	310.3 (pmol/ min/ mg protein)	295.7 (pmol/ min/ mg protein)	100%
Naringenin	69.2	65.3	67.3
Troleandomycin	90.1	84.6	87.4
Quercetin	40.2	33.0	36.6
Ketoconazole	42.7	50.4	46.6
Quinidine	93.5	86.5	90.0

Table 6 Inhibition of paraoxon formation from parathion (200μM) by two human liver microsomal preparations. Values are percent of control activity (mean of duplicate estimations).

Inhibitor (100μM)	Liver M9	Liver M11	Mean
None (control)	210.3 (pmol/ min/ mg protein)	175.5 (pmol/ min/ mg protein)	100%
Metyrapone	71.7	89.2	80.5
α-Naphthoflavone	82.8	84.1	83.5
DEDC	110.2	122.3	116.3
Tolbutamide	89.8	122.7	106.3

DEDC = Diethyldithiocarbamate

Table 7 Inhibition of *p*-nitrophenol formation from parathion (200μM) by two human liver microsomal preparations. Values are percent of control activity (mean of duplicate estimations).

Inhibitor (100μM)	Liver M15	Liver M16	Mean
None (control)	721.5 (pmol/ min/ mg protein)	499.5 (pmol/ min/ mg protein)	100%
Naringenin	53.2	48.1	50.7
Troleandomycin	86.7	86.5	86.6
Quercetin	48.4	45.0	46.7
Ketoconazole	28.2	36.2	32.2
Quinidine	113.0	99.0	106.0

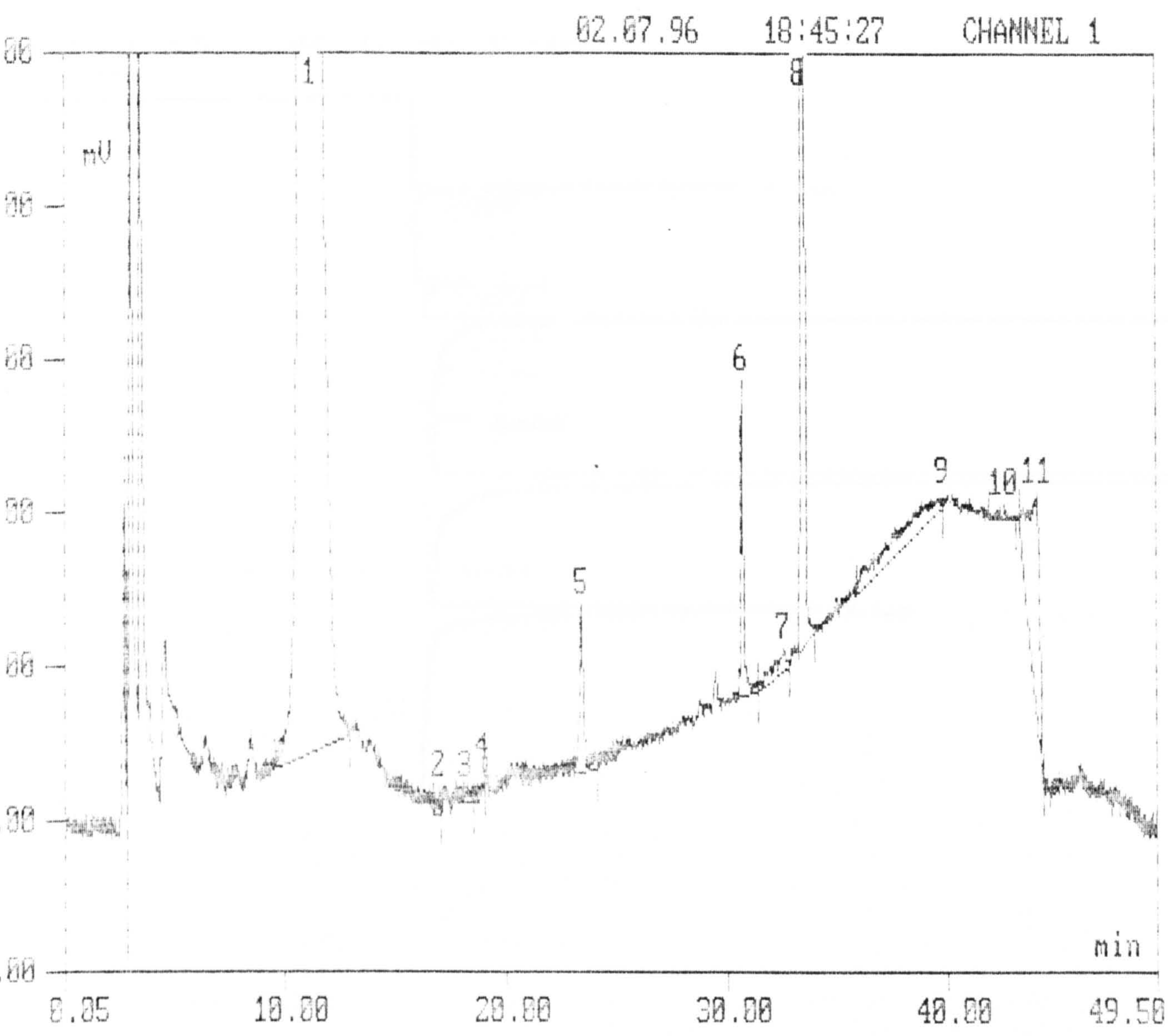
Table 8 Inhibition of *p*-nitrophenol formation from parathion (200μM) by two human liver microsomal preparations. Values are percent of control activity (mean of duplicate estimations).

Inhibitor (100μM)	Liver M9	Liver M11	Mean
None (control)	511.4 (pmol/ min/ mg protein)	578.2 (pmol/ min/ mg protein)	100%
Metyrapone	85.1	76.0	80.6
α-Naphthoflavone	80.5	101.7	91.1
DEDC	118.3	120.5	119.4
Tolbutamide	100.0	115.0	107.5

DEDC = Diethyldithiocarbamate

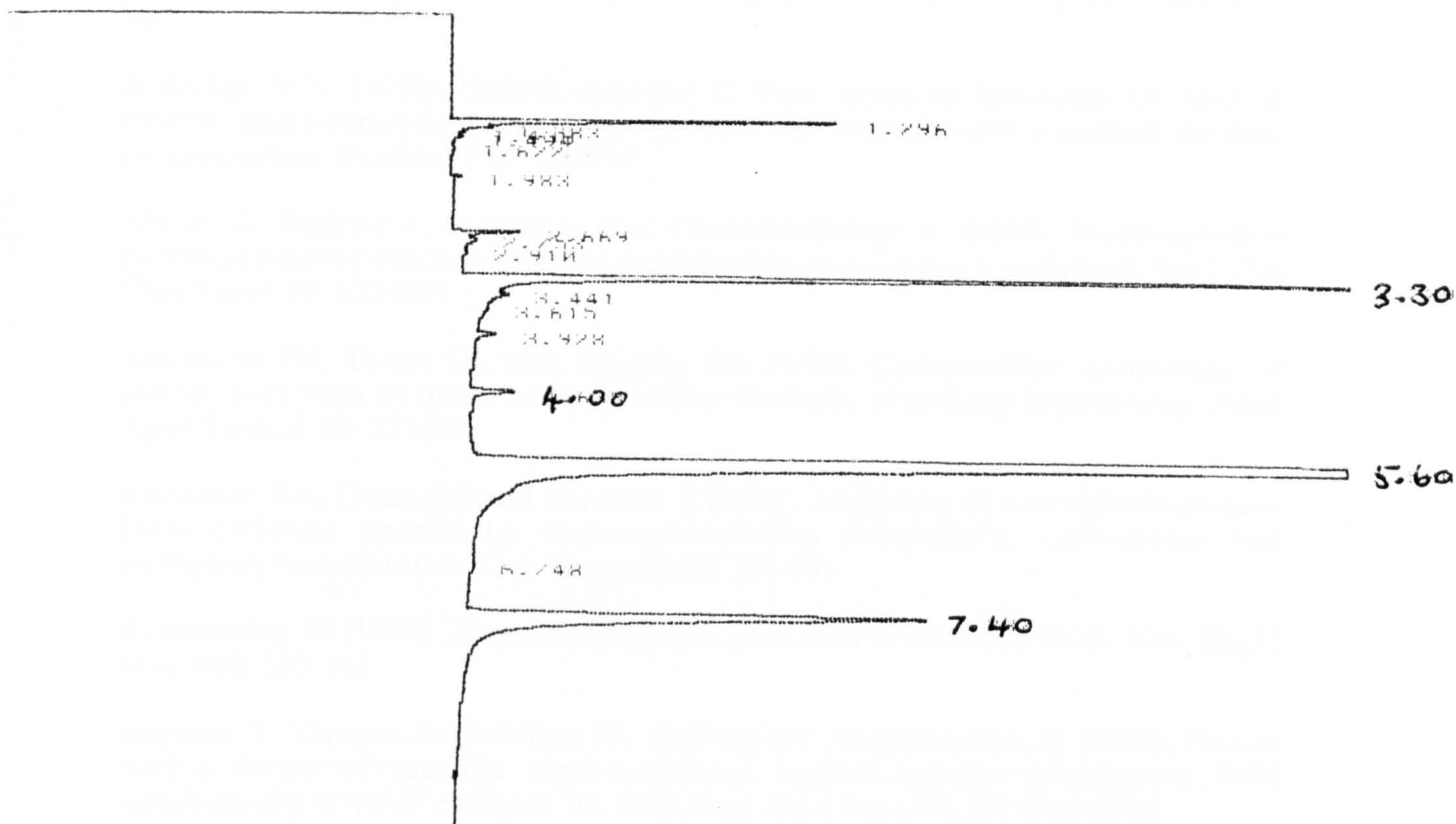
Chapter 15

Appendix V



A typical chromatogram produced following incubation of rat liver microsomes with parathion (200 μ M). The retention times for *p*-nitrophenol, paraoxon and parathion are 23.8min, 30.8min and 33.5min.

RUN # 1475 SEP 30 1991 01:30:41
START



A typical chromatogram produced following incubation of rat or human liver microsomes with nifedipine (100 μ M). The retention times for MI, unknown metabolite, nifedipine and nitrendipine (IS) are 3.3min, 4.0min, 5.6min and 7.4min.

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